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(54) Title: COMPOSITIONS AND METHODS FOR TREATING CANCER USING CYTOTOXIC CD44 ANTIBODY IMMUNO-  
CONJUGATES AND CHEMOTHERAPEUTIC AGENTS

(57) Abstract: The invention relates to the combined use of conjugates of CD44 specific antibodies with cytotoxic compounds and chemotherapeutic agents in cancer therapy, pharmaceutical compositions comprising such compounds and/or chemotherapeutic agents, and methods of cancer treatment. Preferred conjugates contain maytansinoids as cytotoxic compounds, and preferred chemotherapeutic agents are taxanes, epothilones, and vinca alkaloids.

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## Compositions and methods for treating cancer using cytotoxic CD44 antibody immunoconjugates and chemotherapeutic agents

### Technical Field

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The invention relates to the combined use of conjugates of antibodies with cytotoxic compounds and chemotherapeutic agents in cancer therapy, pharmaceutical compositions comprising such compounds and/or chemotherapeutic agents, and methods of cancer treatment.

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### Background Art

There have been numerous attempts to improve the efficacy of antineoplastic drugs by conjugating such drugs to antibodies against tumor-associated antigens in order to elevate local concentration of the drug by targeted delivery to the tumor. Many of these approaches have met limited success, and several reasons have been discussed in the literature to explain the failure. For anticancer drugs acting stoichiometrically, like e.g. doxorubicin or methotrexate, relatively high intracellular concentrations are necessary to exert the required cytotoxicity. These concentrations are thought to be difficult to achieve with many antibody-drug conjugates because of (a) insufficient potency of many common anticancer drugs, (b) low cell surface concentration of antigen targets, (c) inefficient internalization of antigen-antibody complexes into the target cell, and (d) inefficient release of free drug from the conjugate inside the target cell (Chari et al., 1992).

25 Two of the aforementioned drawbacks, namely (a) and (d), have been addressed by the work of Chari and coworkers (Chari et al., 1992; Liu et al., 1996; U.S. Patent No. 5,208,020). They have developed antibody conjugates wherein the antibody is linked to a maytansinoid via a disulfide linkage. Maytansines belong to the class of Ansa macrolide antibiotics, which derive from *Nocardia* sp.. The maytansine ansamitocin P-3, produced by bacterial fermentation, is used as a precursor molecule to manufacture maytansinoid DM1. 30 Maytansine and derivatives act as anti-mitotic agents (inhibitors of tubulin polymerization), similar as vincristine, but with markedly higher potency than vincristine or other established chemotherapeutic agents (DM1 is toxic to cells *in vitro* at  $\sim 10^{-10}$  M

concentration). In contrast to the high cytotoxicity of free maytansinoid, the antibody conjugate has a toxicity which is several orders of magnitude lower on antigen-negative cells compared to antigen-positive cells. The linkage by disulfide bonding has the advantage that these bonds are readily cleaved inside the target cells by intracellular glutathione, releasing highly toxic free drug. This approach has been applied to antibodies against tumor-associated antigens, for example the C242-DM1 conjugate (Liu et al., 1996; Lambert et al., 1998), and HuN901-DM1 (Chari et al., 2000). However, the application of these conjugates is restricted due to the limited expression of the respective target antigens. For example, the antigen recognized by N901 (CD56, N-CAM) is predominantly expressed by tumors of neuroendocrine origin, the expression of the C242 antigen (CanAg) is mostly limited to tumors derived from the GI tract.

There is, therefore, still the need to improve this approach by finding suitable tumor-associated antibodies with favorable antigen expression pattern, high and specific cell surface antigen concentration within the target tissue, and efficient internalization process transporting the antigen-complexed antibody conjugate into the cells.

CD44 is a protein which is expressed in several different isoforms on the surface of a wide variety of cell types. The smallest isoform, standard CD44 (CD44s), which is expressed by a variety of different cells, is thought to mediate cell attachment to extracellular matrix components and may transmit a co-stimulus in lymphocyte and monocyte activation. In contrast, expression of splice variants of CD44 which contain the domain v6 (CD44v6) in the extracellular region, is restricted to a subset of epithelia. The physiological role of CD44v6 is not yet fully understood.

CD44v6, as well as other variant exons (CD44v3, CD44v5, CD44v7/v8, CD44v10) has been shown to be a tumor-associated antigen with a favorable expression pattern in human tumors and normal tissues (Heider et al., 1995; Heider et al., 1996; Dall et al., 1996; Beham-Schmid et al., 1998; Tempfer et al., 1998; Wagner et al., 1998) and has been subject to antibody-based diagnostic and therapeutic approaches, in particular radioimmunotherapy (RIT) of tumors (Verel et al., 2002; Stromer et al., 2000, WO 95/33771, WO 97/21104).

However, a prerequisite for efficient killing of tumor cells by antibody maytansinoid conjugates is sufficient internalization of the target antigen. Only few data on the internalization of CD44 are available. Bazil et Horejsi reported that downregulation of CD44 on leukocytes upon stimulation with PMA is caused by shedding of the antigen rather than by internalization (Bazil et Horejsi, 1992). Shedding of CD44 is also supported by several reports on soluble CD44 in the serum of tumor patients and normal individuals (Sliutz et al., 1995; Guo et al., 1994; Martin et al., 1997). In a recent paper by Aguiar et al. the amount of internalized CD44 on matrix-intact chondrocytes was determined to be approximately 6% in 4 hours (Aguiar et al., 1999). Similar low levels of internalized CD44v6 on tumor cells were found in experiments performed by BIA. Taken together, these data suggest that CD44 receptors are more likely subject to shedding than to internalization, and thus CD44 specific antibodies are not to be regarded as suitable candidates for the maytansinoid conjugate approach. This has been supported by in vitro cell proliferation assays wherein Ab<sub>CD44v6</sub>-DM1 showed only slightly elevated cytotoxicity against antigen-presenting cells as compared to cells lacking the antigen.

It now has been found that CD44 specific antibodies conjugated to highly cytotoxic drugs through a linker which is cleaved under intracellular conditions are very efficient tumor therapeutics in vivo. Thus, such compounds may be advantageously used in cancer therapy.

There is still the need for further improvements. For the antibody maytansinoid conjugates huN901-DM1 and C242-DM1, the combination of these conjugates with the taxanes paclitaxel or docetaxel has been suggested (WO 01/24763).

It has now been unexpectedly found that the combination of a conjugate consisting of a CD44 specific antibody and a cytotoxic agent with a further chemotherapeutic agent shows synergistic effects.

**Disclosure of the Invention**

In particular, the present invention relates to the use of a compound of formula

**A(LB)<sub>n</sub>** (Formula (I))

5 wherein

**A** is an antibody molecule which is specific for CD44;

**L** is a linker moiety;

**B** is a compound which is toxic to cells; and

**n** is a decimal number with  $n = 1$  to 10

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for the preparation of a pharmaceutical composition for the treatment of cancer, wherein said compound is used or is for use in combination with a further chemotherapeutic agent.

In a further aspect, the present invention relates to a method of treatment of cancer, wherein an effective amount of a compound of Formula (I), as defined herein, is

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administered to a patient in need thereof in combination with a further chemotherapeutic agent.

The antibody molecule **A** has a binding specificity for CD44, preferably variant CD44, most preferably CD44v6.

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The term „antibody molecule“ shall encompass complete immunoglobulins as they are produced by lymphocytes and for example present in blood sera, monoclonal antibodies secreted by hybridoma cell lines, polypeptides produced by recombinant expression in host cells which have the binding specificity of immunoglobulins or monoclonal antibodies,

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and molecules which have been derived from such immunoglobulins, monoclonal antibodies, or polypeptides by further processing while retaining their binding specificity.

In particular, the term „antibody molecule“ includes complete immunoglobulins comprising two heavy chains and two light chains, fragments of such immunoglobulins

like Fab, Fab', or F(ab)<sub>2</sub> fragments (Kreitman *et al.*, 1993), recombinantly produced

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polypeptides like chimeric, humanised or fully human antibodies (Breitling *et al.* 1999; Shin *et al.*, 1989; Güssow *et al.* Seemann, 1991, Winter *et al.*, 1994, EP 0 239 400; EP 0 519 596; WO 90/07861 EP 0 368 684; EP 0 438 310; WO 92/07075; WO 92/22653; EP 0 680 040; EP 0 451 216), single chain antibodies (scFv, Johnson *et al.* Bird, 1991),

multimeric antibodies (Kortt et al., 2001) like diabodies, triabodies, or tetrabodies, and the like. Today, antibodies may also be produced without immunising a laboratory animal, e.g. by phage display methods (Aujame et al., 1997; US 5,885,793; US 5,969,108; US 6,300,064; US 6,248,516, US 6,291,158). Fully human antibodies may be produced using  
5 transgenic mice carrying functional human Ig genes (EP 0 438 474 ; EP 0 463 151; EP 0 546 073). From the aforementioned literature references, the expert knows how to produce these types of antibody molecules, employing state of the art methods like automated peptide and nucleic acid synthesis, laboratory animal immunisation, hybridoma technologies, polymerase chain reaction (PCR), vector and expression technologies, host  
10 cell culture, and protein purification methods. In the following, the terms „antibody“ and „antibody molecule“ are used interchangeably.

„Specific for CD44“ shall mean that the antibody molecule has specific binding affinity for an epitope present in CD44. In a preferred embodiment, the antibody molecule of the  
15 invention has a binding specificity for the amino acid sequence coded by variant exon v6 of the human CD44 gene. The sequence of variant exon v6 as well as of the other variant exons is known in the art (Screaton *et al.*, 1992; Tölg *et al.*, 1993; Hofmann *et al.*, 1991). A preferred antibody molecule of the invention specifically binds to peptides or polypeptides having or containing the amino acid sequence SEQ ID NO: 1 of the  
20 accompanying sequence listing, or an allelic variant of said sequence. Preferably, said antibody molecule has binding specificity for an epitope within said sequence. More preferably, the antibody molecule specifically binds to a peptide having the amino acid sequence SEQ ID NO: 2, even more preferably having the amino acid sequence SEQ ID NO: 3. Such antibody molecules may be easily produced with methods known in the art  
25 (WO 95/33771, WO 97/21104), e.g. by immunising laboratory animals with chemically synthesised peptides having the aforementioned sequences, e.g. bound to a hapten, or immunising with a recombinantly produced fusion protein including said sequences, and proceeding according to methods known in the art (Harlow 1988; Catty 1988; Koopman *et al.*, 1993; Heider *et al.*, 1993).

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Preferably, an antibody molecule to be used for the present invention is the murine monoclonal antibody with the designation VFF-18 which is produced by a hybridoma cell line which has been deposited on 07 June 1994 under the accession number DSM

ACC2174 with the DSM-Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Deutschland/Germany. Also preferred are Fab, Fab', or F(ab)<sub>2</sub> fragments of said monoclonal antibody VFF-18. In another preferred embodiment, the antibody molecule is a humanised recombinant antibody, wherein the complementarity determining regions (CDR's) of VFF-18 have been grafted into the respective genes of human immunoglobulin heavy and light chains.

"Complementarity determining regions" of a monoclonal antibody are understood to be those amino acid sequences involved in specific antigen binding according to Kabat *et al.*, 1991, in connection with Chothia and Lesk, 1987.

In another preferred embodiment, appropriate framework residues of such a CDR-grafted antibody are reverted to murine residues to improve binding affinity. From methods pertinent to the art, the experts know how to obtain the CDR's of VFF-18, starting with the aforementioned hybridoma with the accession number DSM ACC2174, to choose and obtain appropriate human immunoglobulin genes, to graft the CDR's into these genes, to modify selected framework residues, to express the CDR-grafted antibody in appropriate host cells, e.g. Chinese hamster ovary (CHO) cells, and to test the resulting recombinant antibodies for binding affinity and specificity (see e.g. literature references above). In another preferred embodiment of the invention, the antibody molecule is a recombinant antibody having the CDR's of the antibody VFF-18. Preferably, such a recombinant antibody is a humanised antibody and is a complete immunoglobulin consisting of two complete light and two complete heavy chains. In another preferred embodiment of the invention, the antibody molecule is a recombinant antibody having the same idiotype as the antibody VFF-18. In another preferred embodiment of the invention, the antibody molecule is a recombinant antibody binding to the same epitope as the antibody VFF-18.

In a particular preferred embodiment, the antibody molecule A is an antibody comprising light chains having the amino acid sequence SEQ ID NO: 4, and heavy chains having the amino acid sequence SEQ ID NO: 6. This antibody is called BIWA 4. It is a humanised version of antibody VFF-18 mentioned above, having the complementary determining regions of the murine monoclonal antibody VFF-18 in a completely human framework, and human constant regions. It is therefore an antibody of very low immunogenicity in

man, which is a favorable trait. However, as it has no murine framework residues to optimise antigen binding, it has a significantly lower antigen binding affinity as its parent antibody VFF-18, and therefore would not have been regarded as a good candidate for a therapeutic drug. Unexpectedly, it has been found that BIWA 4, despite its poor binding affinity, has a very favorable biodistribution and tumor uptake in vivo, making it superior to other humanised versions of VFF-18 with higher binding affinity. In a further preferred embodiment, the antibody molecule A is an antibody comprising light chains having the amino acid sequence SEQ ID NO: 8, and heavy chains having amino acid sequence SEQ ID NO: 6. This antibody is called BIWA 8 and has higher binding affinity than BIWA 4.

These antibodies may be produced as follows. Nucleic acid molecules coding for the light chain and the heavy chain may be synthesised chemically and enzymatically by standard methods. First, suitable oligonucleotides can be synthesized with methods known in the art (e.g. Gait MJ, 1984), which can be used to produce a synthetic gene. Methods to generate synthetic genes from oligonucleotides are known in the art (e.g. Stemmer et al. 1995; Ye et al. 1992; Hayden et Mandecki 1988; Frank et al. 1987). Preferably, the nucleic acid molecules encoding the light and heavy chains of BIWA 4 have the nucleotide sequences of SEQ ID NO: 5 and SEQ ID NO: 7, respectively. These sequences include sequences coding for leader peptides which are cleaved by the host cell (SEQ ID NO: 5: the first 60 nucleotides; SEQ ID NO: 7: the first 57 nucleotides). In a further embodiment, the nucleic acid molecules encoding the light and heavy chains of an antibody molecule according to the invention have the nucleotide sequences of SEQ ID NO: 9 and SEQ ID NO: 7, respectively. These nucleic acid molecules encoding the antibody heavy and light chains then may be cloned into an expression vector (either both chains in one vector molecule, or each chain into a separate vector molecule), which then is introduced into a host cell. Expression vectors suitable for immunoglobulin expression in prokaryotic or eukaryotic host cells and methods of introduction of vectors into host cells are well-known in the art. In general, the immunoglobulin gene therein is in functional connection with a suitable promoter, like for example a human cytomegalovirus (CMV) promoter, hamster ubiquitin promoter (WO 97/15664), or a simian virus SV40 promoter located upstream of the Ig gene. For termination of transcription, a suitable termination/polyadenylation site like that of the bovine growth hormone or SV40 may be employed. Furthermore, an enhancer sequence may be included, like the CMV or SV40 enhancer. Usually, the expression



vector furthermore contains selection marker genes like the dihydrofolate reductase (DHFR), glutamine synthetase, adenosine deaminase, adenylate deaminase genes, or the neomycin, bleomycin, or puromycin resistance genes. A variety of expression vectors are commercially available from companies such as Stratagene, La Jolla, CA; Invitrogen, Carlsbad, CA; Promega, Madison, WI or BD Biosciences Clontech, Palo Alto, CA. For example, expression vectors pAD-CMV1 (NCBI GenBank Accession No. A32111) or pAD-CMV19 (NCBI GenBank Accession No. A32110) may be used for expression. The host cell preferably is a mammalian host cell, e.g. a COS, CHO, or BHK cell, more preferably a Chinese hamster ovary (CHO) cell, e.g. a CHO-DUKX (Urlaub and Chasin, 1980), CHO-DG44 (Urlaub et al., 1983), or CHO-K1 (ATCC CCL-61) cell. The host cell then is cultured in a suitable culture medium under conditions where the antibody is produced, and the antibody is then isolated from the culture according to standard procedures. Procedures for production of antibodies from recombinant DNA in host cells and respective expression vectors are well-known in the art (see e.g. WO 94/11523, WO 97/9351, EP 0 481 790, EP 0 669 986).

In order to link the antibody molecule **A** to the compound **B** which is toxic to cells, a linking moiety **L** is used. In the most simple case, the linking moiety **L** is a chemical bond, preferably a covalent bond which is cleaved under intracellular conditions. In one embodiment of the invention, the bond is between a sulfur atom present in the antibody molecule, e.g. in the side chain of a cystein residue, and another sulfur atom present in the toxic compound. In another embodiment, the linking moiety **L** consists of one or more atoms or chemical groups. Suitable linking groups are well known in the art and include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Preferred are disulfide groups and thioether groups.

Conjugates of the antibody molecules of the invention and toxic compound can be formed using any techniques presently known or later developed. The toxic compound can be modified to yield a free amino group and then linked to the antibody molecule via an acid-labile linker, or a photolabile linker. The toxic compound can be condensed with a peptide and subsequently linked to an antibody molecule to produce a peptidase-labile linker. The toxic compound can be treated to yield a primary hydroxyl group, which can be succinylated and linked to an antibody molecule to produce a conjugate that can be cleaved

by intracellular esterases to liberate free drug. Most preferably, the toxic compound is treated to create a free or protected thiol group, and then one or many disulfide or thiol-containing toxic compounds are covalently linked to the antibody molecule via disulfide bond(s).

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For example, antibody molecules can be modified with crosslinking reagents such as N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), 4-succinimidyl-oxycarbonyl- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)-toluene (SMPT), N-succinimidyl-3-(2-pyridyldithio)-butyrate (SDPB), N-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP), N-succinimidyl-5-(2-pyridyldithio)pentanoate, 2-iminothiolane, or acetylsuccinic anhydride by known methods. See, Carlsson et al, 1978; Blattler et al 1985; Lambert et al, 1983; Klotz et al, 1962; Liu et al, 1979; Blakey and Thorpe, 1988; Worrell et al, 1986. In a preferred embodiment, the linker moiety is a 5-thiopentanoate or 4-thiopentanoate derived from SPP. The antibody molecule containing free or protected thiol groups thus derived is then reacted with a disulfide- or thiol-containing toxic compound to produce conjugates. The conjugates can be purified by HPLC or by gel filtration.

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„Toxic“ is a compound that inhibits or prevents function of cells and/or causes cell destruction. Toxic compounds used for coupling may act either cytostatic or cytotoxic and lead to cell cycle arrest or cell death. These compounds may act at different stages during the cell cycle, e.g. by interference with nucleic acid synthesis, inactivation of nucleic acids, or by binding to tubulin.

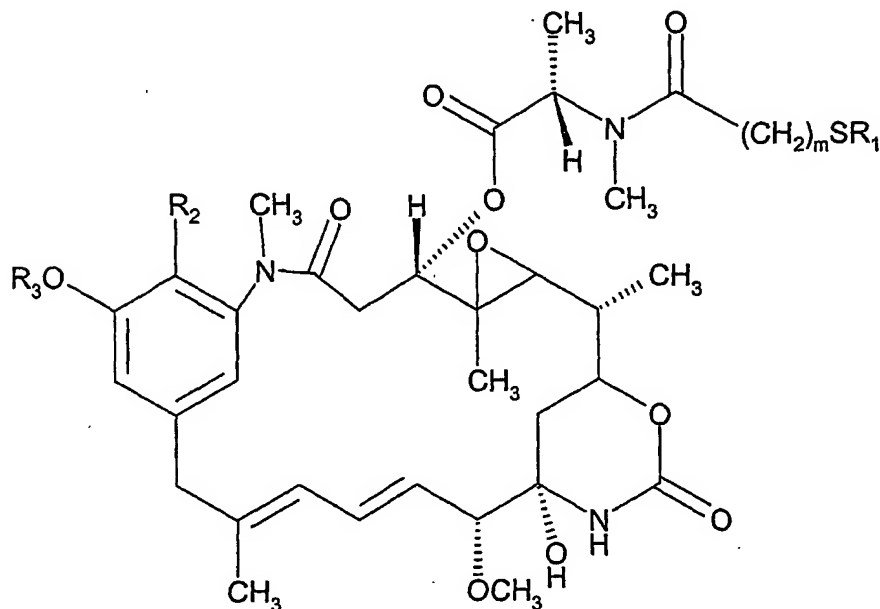
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In a preferred embodiment, the compound B present in  $A(LB)_n$  which is toxic to cells is a maytansinoid, i.e. a derivative of maytansine (CAS 35846538). In a preferred embodiment, it is a C-3 ester of maytansinol. Maytansinoids suitable for conjugating to antibodies for use in cancer therapy, including preparation of said maytansinoids and their linkage to antibody molecules, have been described by Chari and Coworkers (Chari et al., 1992; Liu et al., 1996; U.S. Patent No. 5,208,020). These maytansinoids may be used for the present invention. In a preferred embodiment, the toxic compound is N<sup>2'</sup>-deacetyl- N<sup>2'</sup>-(3-mercapto-1-oxopropyl)-Maytansine (CAS Number 139504-50-0), also referred to as DM1. Preferably, said maytansinoid is a maytansinol derivative linked to the antibody molecule via a disulfide bridge at the C-3 position of maytansinol. In a particularly preferred

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embodiment, the antibody/maytansinoid conjugate may be prepared from a maytansinoid of formula

Formula (II)



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wherein

$R_1$  represents H or  $SR_4$ , wherein  $R_4$  represents methyl, ethyl, linear alkyl, branched alkyl, cyclic alkyl, simple or substituted aryl, or heterocyclic;

10  $R_2$  represents Cl or H;

$R_3$  represents H or  $CH_3$ ; and

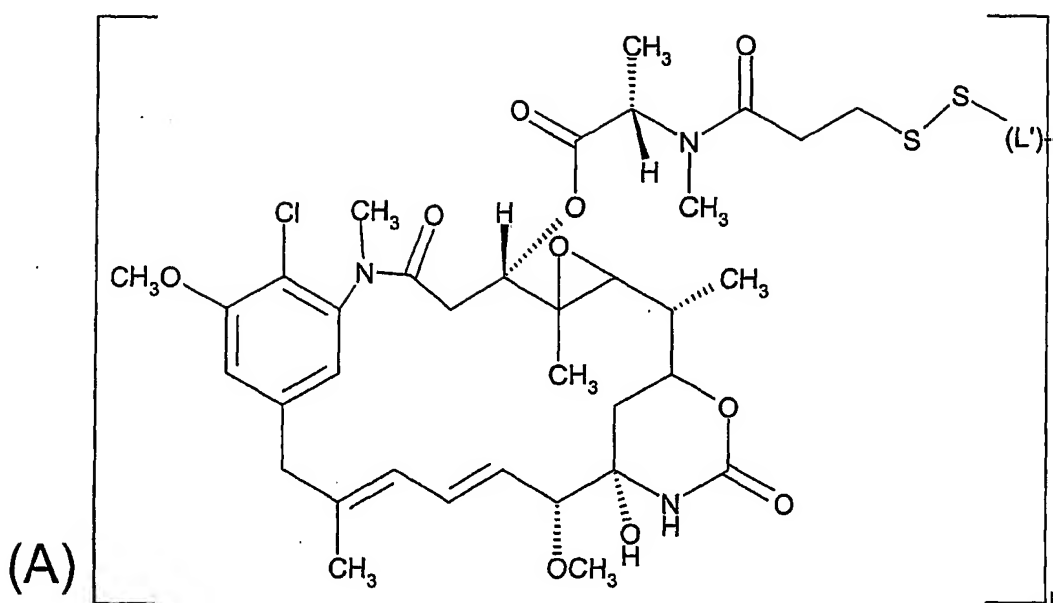
$m$  represents 1, 2, or 3.

Preferably,  $R_1$  is H,  $CH_3$ , or  $SCH_3$ ,  $R_2$  is Cl,  $R_3$  is  $CH_3$ , and  $m = 2$ .

15 The compound with  $R_1 = H$ ,  $R_2 = Cl$ ,  $R_3 = CH_3$ , and  $m = 2$  is designated DM1 in the literature.

In a preferred embodiment, the compound of the invention has the formula

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(Formula III)

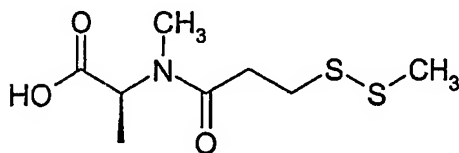
wherein

- 5 A is an antibody molecule which is specific for CD44, preferably specific for the variant exon v6, preferably specific for the amino acid sequence SEQ ID NO: 3;  
 (L') is an optional linker moiety  
 p is a decimal number with  $p = 1$  to 10

- 10 Preferably,  $p$  is 3 to 4, more preferably about 3.5.

Methods for preparing such maytansinoids are known in the art (see in particular US 5,208,020, Example 1). Conveniently, in a first step the maytansinoid C-3 ester ansamitocin P3 may be produced by bacterial fermentation (US 4,356,265; US 4,450,234; 15 WO 01/77360) of microorganisms belonging to the genus *Nocardia* or *Actinosynnema*, e.g. ATCC 31565, ATCC 31281. Ansamitocin P3 may be extracted from the culture using organic solvents like ethyl acetate or toluene, and further purified by adsorption chromatography using e.g. silica gel. It may then be reduced to maytansinol using LiAlH<sub>4</sub> (US 4,360,462) or, as suggested more recently (WO 02/16368), LiAl(OMe)<sub>3</sub>H or other 20 LiAl or NaAl hydrides. The maytansinol may then be esterified at the C-3 position with N-methyl-L-alanine or N-methyl-L-cysteine derivatives to yield a disulfide-containing maytansinoid (US 5,208,020; US 5,416,064; US 6,333,410), for example using

dicyclohexylcarbodiimide(DCC) and catalytic amounts of zinc chloride (US 4,137,230; US 4,260,609). In a preferred embodiment, the maytansinol is esterified with the compound N-methyl-N-(3-methyldithiopropionyl)-L-alanine of formula



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to yield the maytansinoid of Formula (II) with  $R_1 = SR_4$ ,  $R_4 = CH_3$ ,  $R_2 = Cl$ ,  $R_3 = CH_3$ , and  $m = 2$ . Manufacture of N-methyl-L-alanine or N-methyl-L-cysteine derivatives is disclosed for example in US 5,208,020 and WO 02/22554.

- 10 The free thiol group may then be released by cleavage of the disulfide bond with dithiothreitol (DTT), to yield e.g. DM1.

Upon intracellular cleavage, the free toxic compound is released. The free drug released from the compound  $A(LB)_n$  may have the formula  $B-X$ , wherein  $X$  is an atom or a chemical group, depending on the nature of the cleaving reaction. Preferably,  $X$  is a hydrogen atom, as for example when the linker moiety is just a covalent bond between two sulfur atoms, or a hydroxyl group. The cleavage site may also be within the linker moiety if the linker moiety is a chemical group, generating free drug of formula  $B-L'-X$  upon cleavage, wherein  $X$  is an atom or a chemical group, depending on the nature of the cleaving reaction. Preferably,  $X$  is a hydrogen atom or a hydroxyl group. The free drug released intracellularly may also contain parts (amino acid or peptidic residues) of the antibody molecule, if the linker is stable, but the antibody molecule is degraded.

In a preferred embodiment, the compound of Formula (I) is less toxic than the toxic compound  $B$ ,  $B-X$  or  $B-L'-X$  released upon intracellular cleavage. Methods of testing cytotoxicity in vitro are known in the art (Goldmacher et al., 1985; Goldmacher et al., 1986; see also US 5,208,020, Example 2). Preferably, the compound (I) is 10 times or more, more preferably 100 times or more, or even 1000 times or more less toxic than the free drug released upon cleavage.

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Preferably, antibody molecule/maytansinoid conjugates are those that are joined via a disulfide bond, as discussed above, that are capable of delivering maytansinoid molecules. Such cell binding conjugates are prepared by known methods such as modifying monoclonal antibodies with succinimidyl pyridyl-dithiopropionate (SPDP) or pentanoate (SPP; N-succinimidyl-4-(2-pyridyldithio)pentanoate, or N-succinimidyl-5-(2-pyridyldithio)pentanoate) (Carlsson et al, 1978). The resulting thiopyridyl group is then displaced by treatment with thiol-containing maytansinoids to produce disulfide linked conjugates. Alternatively, in the case of the arylthiomaytansinoids, the formation of the antibody conjugate is effected by direct displacement of the aryl-thiol of the maytansinoid by sulfhydryl groups previously introduced into antibody molecules. Conjugates containing 1 to 10 maytansinoid drugs linked via a disulfide bridge are readily prepared by either method. In this context, it is understood that the decimal number  $n$  in the formula  $A(LB)_n$  is an average number as not all conjugate molecules of a given preparation may have the identical integer of  $LB$  residues attached to the antibody molecule.

More specifically, a solution of the dithiopyridyl modified antibody at a concentration of 1 mg/ml in 0.1 M potassium phosphate buffer, at pH 7.0 containing 1 mM EDTA is treated with the thiol-containing maytansinoid (1.25 molar equivalent/dithiopyridyl group). The release of pyridine-2-thione from the modified antibody is monitored spectrophotometrically at 343nm and is complete in about 30 min. The antibody-maytansinoid conjugate is purified and freed of unreacted drug and other low molecular weight material by gel filtration through a column of Sephadex G-25. The number of maytansinoids bound per antibody molecule can be determined by measuring the ratio of the absorbance at 252 nm and 280 nm. An average of 1-10 maytansinoid molecules/antibody molecule can be linked via disulfide bonds by this method.

In a preferred aspect, the present invention relates to a conjugate of a CD44v6 specific antibody molecule and a maytansinoid. Herein, „CD44v6 specific“ shall mean that the antibody has specific binding affinity to an epitope which is present in a peptide having the amino acid sequence encoded by variant exon v6 of CD44, preferably human CD44. A preferred antibody molecule of the invention specifically binds to peptides or polypeptides having or containing the amino acid sequence SEQ ID NO: 1 of the accompanying sequence listing, or an allelic variant of said sequence. Preferably, said antibody molecule

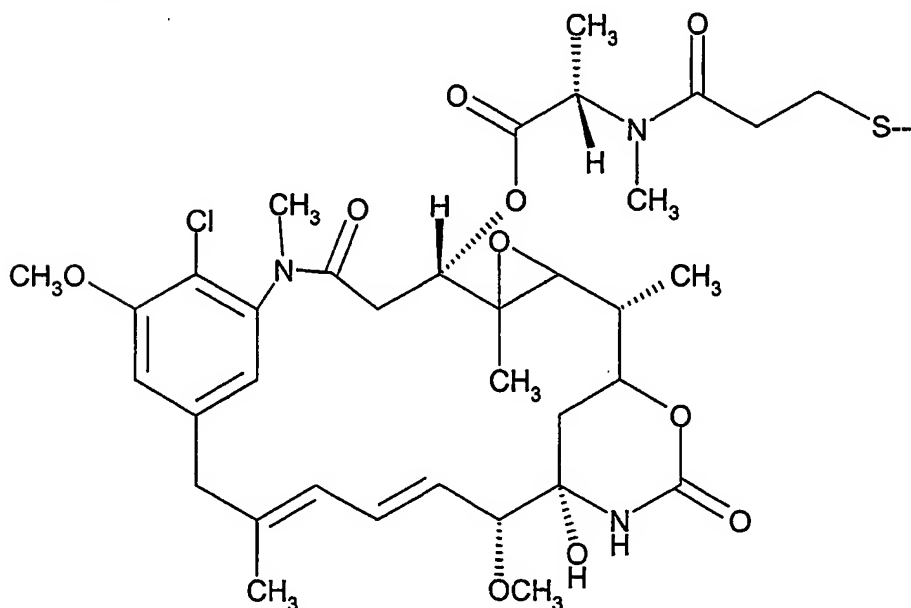
has binding specificity for an epitope within said sequence. More preferably, the antibody molecule specifically binds to a peptide having the amino acid sequence SEQ ID NO: 2, even more preferably having the amino acid sequence SEQ ID NO: 3.

- 5 Preferably, the antibody molecule in said conjugate is the monoclonal antibody VFF-18 (DSM ACC2174) or a recombinant antibody having the complementary determining regions (CDRs) of VFF-18. More preferably, the said antibody comprises light chains having the amino acid sequence SEQ ID NO: 4, or, alternatively, SEQ ID NO: 8, and heavy chains having the amino acid sequence SEQ ID NO: 6.

10

The maytansinoid is preferably linked to the antibody by a disulfide moiety and has the formula

Formula (IV)



15

wherein the link to the antibody is through the sulfur atom shown in formula IV to a second sulfur atom present in the antibody molecule. To create such a sulfur atom available for bonding, an antibody molecule may be modified by introduction of a suitable linker as outlined above. Preferably, the maytansinoid is linked to the antibody molecule through a  $-S-CH_2CH_2-CO-$ , a  $-S-CH_2CH_2CH_2CH_2-CO-$ , or a  $-S-CH(CH_3)CH_2CH_2-CO-$  group. The sulfur atom in such a linker group forms the disulfide bond with the

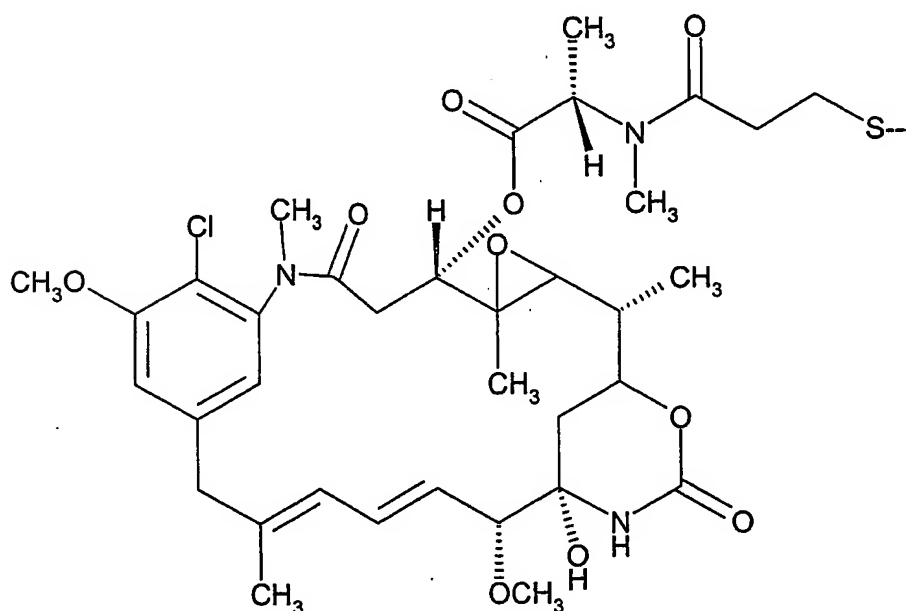
20

maytansinoid, while the carbonyl function may be bonded to an amino function present on the side chain of an amino acid residue of the antibody molecule.

That way, one or more maytansinoid residues may be linked to an antibody molecule.

5 Preferably, 3 to 4 maytansinoid residues are linked to an antibody molecule.

Most preferred is a conjugate of a CD44v6 specific antibody molecule and a maytansinoid, wherein the antibody comprises light chains having the amino acid sequence SEQ ID NO: 4, and heavy chains having the amino acid sequence SEQ ID NO: 6, and wherein the  
10 maytansinoid has the formula



(Formula IV)

and is linked to the antibody through a disulfide bond. Preferably, the linking group is -S-  
15  $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-CO-}$  or  $\text{-S-CH(CH}_3\text{)CH}_2\text{CH}_2\text{-CO-}$ , and the number of maytansinoid residues bound per antibody molecule is 3 to 4.

The conjugate or compound of Formula (I), as defined herein, is preferably formulated into a pharmaceutical composition comprising such conjugate or compound, preferably  
20 together with a pharmaceutically acceptable carrier, excipient, or diluent.



Suitable pharmaceutically acceptable carriers, diluents, and excipients are well known and can be determined by those of skill in the art as the clinical situation warrants. In general, the conjugate may be formulated in form of a buffered aqueous solution, using a physiologically acceptable buffer like phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l  $\text{Na}_2\text{HPO}_4$ , 0.24 g/l  $\text{KH}_2\text{PO}_4$  in distilled water, adjusted to pH 7.4 with aqueous HCl), which may contain additional components for solubilisation, stabilisation, and/or conservation, e.g. serum albumin, ethylenediaminetetraacetate (EDTA), benzyl alcohol, or detergents like polyoxyethylenesorbitan monolaurate (Tween 20<sup>TM</sup>). Examples of suitable carriers, diluents and/or excipients include: (1) Dulbecco's phosphate buffered saline, pH about 7.4, containing about 1 mg/ml to 25 mg/ml human serum albumin, (2) 0.9% saline (0.9% w/v NaCl), and (3) 5% (w/v) dextrose. The formulation may also be in form of a freeze-dried powder which may be reconstituted with water or buffer before administration. Such lyophilisates may contain an bulking agent like, for example, mannitol.

15

For clinical treatment of cancer, the compound of Formula (I) according to the invention, in particular the conjugate of a CD44v6 specific antibody molecule and a maytansinoid, will be supplied as solutions that are tested for sterility and for endotoxin levels. Examples of suitable protocols of conjugate administration are as follows. Conjugates may be given weekly for 1 to 6 weeks either as an i.v. bolus, or as a continuous infusion for 5 days. Bolus doses can be given in 50 to 100 ml of isotonic saline to which 5 to 10 ml of human serum albumin has been added. Continuous infusions can be given in 250 to 500 ml of isotonic saline, to which 25 to 50 ml of human serum albumin has been added, per 24 hour period. Dosages will be 10 mg to 400  $\text{mg/m}^2$  of body surface area per application. The dose applied to the patient per administration has to be high enough to be effective, but must be below the dose limiting toxicity (DLT). In general, a sufficiently well tolerated dose below DLT will be considered maximum tolerated dose (MTD). The expert knows how to determine the MTD (Lambert et al., 1998). For weekly administrations, the MTD can be expected to be in the range of 50 to 200  $\text{mg/m}^2$ . Alternatively, intervals between applications may be longer, e.g. two to four weeks, preferably three weeks. In this case, the MTD can be expected to be in the range of 100 to 300  $\text{mg/m}^2$ . Alternatively, application may be in 5 daily doses, followed by a break of several weeks after which treatment may be repeated. In this case, the MTD per administration can be expected to be lower than 100

mg/m<sup>2</sup>. For example, conjugates can be administered as a single i.v. infusion with a rate of 3 mg/min every 21 days.

„Chemotherapeutic agent“, in the context of this invention, shall mean a chemical  
5 compound which inhibits or kills growing cells and which can be used or is approved for  
use in the treatment of cancer. As the compound of Formula (I), in particular the conjugate  
of a CD44v6 specific antibody molecule and a maytansinoid itself is a chemotherapeutic  
agent in that sense, it is understood that the present invention relates to the combination of  
such a compound of Formula (I) or conjugate with a second, structurally different  
10 chemotherapeutic agent. Preferably, the chemotherapeutic agent to be combined with a  
compound of Formula (I), or conjugate as defined above, is not itself an immunoconjugate.  
Preferred chemotherapeutic agents for such a combination are cytostatic agents which  
prevent, disturb, disrupt or delay cell division at the level of nuclear division or cell plasma  
division. Preferably, the chemotherapeutic agent is an anti-mitotic agent, in particular it is  
15 a spindle poison acting by interfering with microtubule function, causing mitotic arrest.  
Such agents may be classified in two groups, those like taxanes that stabilise microtubule  
lattices or those, among them the vinca alkaloids, that preferentially form alternate lattice  
contact and polymers at microtubule ends and thus destabilise microtubules (Future  
Oncology 6: 1421-1456, 2002; Goodsell, 2000). Both types of spindle poisons are  
20 comprised in the present invention.

Hence, preferred chemotherapeutic agents are those which bind to tubulin. Preferred  
agents stabilising microtubules are taxanes, in particular docetaxel or paclitaxel, and  
epothilones, in particular epothilone A, B, C, D, E, and F. Preferred agents which  
25 destabilise microtubules are vinca alkaloids, in particular vinblastine, vincristine,  
vindesine, vinflunine, and vinorelbine.

Taxanes are anti-mitotic agents isolated from yew trees of the genus *taxus* and their  
naturally occurring, semi-synthetically, or synthetically obtained derivatives (Future  
30 Oncology 6: 1421-1456, 2002; The Oncologist 5: 345-346, 2000). Paclitaxel (Taxol<sup>®</sup>,  
Suffness [Ed.], 1995; Wani et al., 1971; Holton et al., 1994; Runowicz et al., 1993) is an  
approved anti-cancer drug formulated as a non-aqueous solution (in polyoxyethylated  
castor oil/ethanol) intended for dilution with a suitable parenteral fluid for intravenous

infusion at doses of 15-275 mg/m<sup>2</sup> applied as 1-, 6-, or 24 hour infusions. For combination therapy with another chemotherapeutic drug, an infusion at a dose of 135 or 175 mg/m<sup>2</sup> over a period of 3 or 24 hours is recommended. Docetaxel (Taxotere<sup>®</sup>; EP 0 253 738; US 4,814,470; Mangatal et al., 1989; Denis et al., 1991; Burris et al., 1993) is also an approved  
5 anti-neoplastic taxane drug. Recommended administration is at a dose of 60 to 100 mg/m<sup>2</sup> infused intravenously over a period of 1 week. Another taxane useful in the context of the present invention is RPR-116258A (Goetz et al., 2001). Other taxanes known in the art which are useful for cancer treatment may be used for the present invention as well. Such taxanes are disclosed for example in WO 01/70718, WO 01/57028, WO 01/57027, WO  
10 01/56564, WO 01/57030, WO 01/57029, WO 01/57031, WO 01/56565, WO 01/57032, WO 01/27115, WO 01/55126, WO 00/50059, US 6,002,023, WO 99/52887, US 5,998,656, WO 99/32473, US 5,892,063, WO 99/14209, US 5,763,477, WO 98/14187, WO 98/08833, WO 98/02426, US 5,705,508, US 5,703,247, WO 97/43291, WO 97/10234, WO 97/09979, EP 747 372, WO 96/21658, GB 2296239, WO 96/14308, WO 96/03394, EP 693 485, GB  
15 2289277, WO 95/25728, WO 95/24402, WO 95/11020, EP 639 577, WO 94/27984, US 5,367,086, WO 94/20088, WO 95/07900, WO 94/21651, WO 94/21252, WO 94/21251, WO 94/21250, EP 617 034, WO 94/17052, WO 94/17050, WO 94/25449, WO 94/15599, EP 604 910, WO 94/12484, WO 94/10997, WO 94/08984, EP 668 762, WO 94/11362, WO 94/01425, WO 93/23389, EP 534 709, EP 534 708, and EP 534 707.

20

Other compounds that can be used in the invention are those that act through a taxane mechanism. Compounds that act through a taxane mechanism include compounds that have the ability to exert microtubule-stabilizing effects and cytotoxic activity against proliferating cells, such as tumor cells or other hyperproliferative cellular diseases. Such  
25 compounds include, for example, epothilone compounds, such as, for example, epothilone A, B, C, D, E, F, BMS-247550, BMS- 310705, and derivatives thereof. Epothilone compounds are spindle poisons produced by certain myxobacteria and their naturally occurring, semi-synthetically, or synthetically obtained derivatives. Epothilone compounds and derivatives thereof are known in the art and are described, for example, in U. S. Patent  
30 Nos. 6,121,029, 6,117,659, 6,096,757, 6,043,372, 5,969,145, 5,886,026, WO 97/19086, WO 98/08849, WO 98/22461, WO 98/25929, WO 98/38192, WO 99/01124, WO 99/02514, WO 99/03848, WO 99/07692, WO 99/27890, and WO 99/28324. Particularly preferred is epothilone B (CAS No. 152044-54-7; WO 98/25929; White et al., 1999;

Valluri et al., 2001; Muhlrad et al., 1997; Chou et al., 1998; Chen et al., 2000) which may be applied at doses between 0.3 and 3.6 mg/m<sup>2</sup>, more preferably between 0.3 and 2.5 mg/m<sup>2</sup>. Also preferred is the epothilone B analogue BMS-247550 disclosed by Yamaguchi et al., 2002, and Lee et al., 2001. Also preferred is the epothilone A analogue BMS-310705  
5 (Vite et al., 2002; Mekhail et al., 2002). Also preferred is epothilone A, the synthesis of which is described by Zhu et Panek, 2000, and epothilone E, the synthesis of which is described by Nicolaou et al., 1999. Also preferred is epothilone D (US 6,204,388; US 6,303,342; Wang et al., 2001; Chou et al., 1998) which may be applied at a dose of 10-20 mg/m<sup>2</sup>.

10

Vinca alkaloids are spindle poisons produced by plants of the genus *catharanthus* (formerly *vinca* Linn.), in particular *catharanthus roseus*, and their naturally occurring, semi-synthetically, or synthetically obtained derivatives. Vinblastine, e.g. in the form of its sulfate salt, is an approved anti-cancer drug (Gorman et al., 1959; US 3,097,137; US  
15 3,225,030; Sieber et al., 1976; Lu et Meistrich, 1979; Muhtadi et Afifi, 1992). The sulfate salt may be formulated as pure substance which is dissolved in physiological saline before administration and may be applied as a intravenous bolus injection at a dose of 3 to 18.5 mg/m<sup>2</sup>, preferably 5.5 to 7.5 mg/m<sup>2</sup> once weekly. Vincristine, e.g. in the form of its sulfate, is also an approved anti-cancer drug (Neus et al., 1964; Sieber et al., 1976; Owellen et  
20 Donigian, 1972; Burns, 1972). The sulfate may be formulated as a solid preparation containing an equal amount of lactose as an excipient and dissolved in isotonic saline before administration. 1 to 2 mg/m<sup>2</sup> of the drug may be applied per week as an intravenous bolus application. Vindesine is a synthetic derivative of vinblastine and also an approved chemotherapeutic agent (DE 2415980; Burnett et al., 1978; Owellen et al., 1977; Krivit et  
25 al., 1979). Vindesine sulfate may be formulated as a powder formulation with mannitol as an excipient (at a ratio of 1:5) and dissolved in water or isotonic saline before administration. 2-4 mg/m<sup>2</sup> may be applied as a weekly intravenous bolus injection.

Vinorelbine is a semi-synthetic vinca alkaloid also approved for cancer therapy (US 4,307,100; Mangeney et al., 1979; Rahmani et al., 1987; Marty et al., 1989). It may be  
30 formulated as a vinorelbine bis[(R,R)-tartrat] in water at a concentration of 10 mg/ml, diluted with isotonic saline or 5% glucose solution before administration, and intravenously infused at a dose of 20 to 30 mg/m<sup>2</sup> per week. Vinflunine (CAS 162652-95-1; Decosterc et al., 1999) and navelbine (Van-den-Berge et al., 1993) may also be used for

the present invention. In the art, further vinca alkaloids are known which may be used in connection with the present invention (WO 99/62912; WO 98/45301; US 5,369,111; US 5,888,537; US 5,891,724; 5,676,978; US 4,096,148).

5 Further microtubule-destabilising agents to be used in the context of the present invention are 5,6-dihydroindolo[2,1-a]isoquinoline derivatives (Goldbrunner et al., 1997). Particularly suitable is combretastatin A4-phosphate (Horsman et al., 1998), and its derivatives like hydroxphenastatin (Pettit et al., 2000), or AVE 8062 (Ohsumi et al., 1998). Spongistatins like spongistatin 1, 2, 3, 4, 5, 6, 7, 8, or 9, may be also used (EP 0 608 111; 10 EP 0 632 042; EP 0 634 414). Another tubulin antagonist for use in the present invention is E-7010 (CAS 141430-65-1; Hoshi et Castaner, 1993). Other tubulin antagonists which may be used in the context of the present invention are dolastatins like cemadotin hydrochloride (Mross et al., 1996). Mivobulin isethionate may also be used (De-Ines et al., 1994).

15 The compound of formula (I), in particular the conjugate of a CD44v6 specific antibody molecule and a maytansinoid, may well combined with other chemotherapeutic agents like cryptophycins, camptothecins (in particular, camptothecin, topotecan, irinotecan, 9-aminocamptothecin), or epipodophyllotoxins (in particular, etoposide, or teniposide). Also 20 to be used in connection with the present invention are anthracyclines like doxorubicin and daunorubicin. Furthermore, antibiotics like dactinomycin, plicamycin, mitomycin, bleomycin, and idarubicin may be used. Alkylating agents like cyclophosphamide, mechlorethamine, melphalan, chlorambucil, procarbazine, dacarbazine, altretamine, platinum compounds (in particular, cisplatin, carboplatin, oxaliplatin, iproplatin, 25 ormaplatin, tetraplatin), or nitrosureas like carmustine, lomustine, or semustine may be used as well. Also comprised are methotrexate, purine antagonists like mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, or pyrimidine antagonists like fluorouracil, doxifluridine (5'-deoxy-5-fluorouridine), capecitabine, cytarabine, or azacytidine. In a further preferred embodiment, the chemotherapeutic agent is capecitabine 30 (N4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine; Ishikawa et al., Biol Pharm Bull. 1998 Jul;21(7):713-7).

In the context of this invention, „in combination with“ shall mean that the compound of Formula (I), in particular the conjugate of a CD44v6 specific antibody molecule and a maytansinoid, and the chemotherapeutic agent are administered to the patient in a regimen wherein the patient may profit from the beneficial effect of such a combination. In particular, both drugs are applied to the patient in temporal proximity. In a preferred embodiment, both drugs are applied to the patient within four weeks (28 days). More preferably, both drugs are administered within two weeks (14 days), more preferred within one week (7 days). In a preferred embodiment, the two drugs are administered within two or three days. In another preferred embodiment, the two drugs are administered at the same day, i.e. within 24 hours. In another embodiment, the two drugs are applied within four hours, or two hours, or within one hour. In another embodiment, the two drugs are administered in parallel, i.e. at the same time, or the two administrations are overlapping in time. For example, they may be infused at the same time, or the infusions may be overlapping in time. If the two drugs are administered at the same time, they may be formulated together in one single pharmaceutical preparation, or they may be mixed together immediately before administration from two different pharmaceutical preparations, for example by dissolving or diluting into one single infusion solution. In another embodiment, the two drugs are administered separately, i.e. as two independent pharmaceutical compositions. In one preferred embodiment, administration of the two drugs is in a way that tumour cells within the body of the patient are exposed to effective amounts of both drugs at the same time. In another preferred embodiment, effective amounts of both drugs are present at the site of the tumour at the same time. In another preferred embodiment, effective amounts of both drugs are present in the body of the patient at the same time. The present invention also embraces the use of further agents, which are administered in addition to the combination as defined. This could be, for example, one or more further chemotherapeutic agent(s). It could also be one or more agent(s) applied to prevent, suppress, or ameliorate unwanted side effects of any of the other drugs given. For example, a cytokine stimulating proliferation of leukocytes may be applied to ameliorate the effects of leukopenia or neutropenia.

Dose, route of administration, application scheme, repetition and duration of treatment will in general depend on the nature of the disease (type, grade, and stage of the tumor etc.) and the patient (constitution, age, gender etc.), and will be determined by the medical expert

responsible for the treatment. With respect to the possible doses for the components of the disclosed combination which are described above, it is clear that the medical expert responsible for the treatment will carefully monitor whether any dose-limiting toxicity or other severe side effects occur and undertake the necessary steps to manage those.

5

The present invention is of particular advantage for the treatment of squameous cell carcinomas expressing CD44 antigen, preferably CD44v6. It is in particular suitable for head and neck squameous cell carcinoma, esophagus squameous cell carcinoma, lung squameous cell carcinoma, skin squameous cell carcinoma, or cervix squameous cell carcinoma. Furthermore, it is of particular advantage for the treatment of adenocarcinomas expressing CD44 antigen, preferably CD44v6. It is in particular suitable for breast adenocarcinoma, lung adenocarcinoma, pancreas adenocarcinoma, colon adenocarcinoma, or stomach adenocarcinoma. Besides treatment of clinically apparent malignant disease, therapeutic application according to the invention may be particularly advantageous as an adjuvant to surgical intervention, to treat minimal residual disease.

10  
15

In a further aspect, the present invention relates to a pharmaceutical composition comprising a compound  $A(LB)_n$  or conjugate as defined herein, together with a chemotherapeutic agent as defined herein, and optionally further comprising one or more pharmaceutically acceptable carrier(s), diluent(s), or excipient(s).

20

In a further embodiment, the present invention relates to a kit comprising, in separate pharmaceutical compositions, a compound  $A(LB)_n$  as defined before, in particular a conjugate of a CD44v6 specific antibody molecule and a maytansinoid, and a chemotherapeutic agent as herein defined.

25

In a further embodiment, the present invention relates to the use of a chemotherapeutic agent for the preparation of a pharmaceutical composition for the treatment of cancer, wherein said chemotherapeutic agent is used or is for use in combination with a compound of Formula  $A(LB)_n$  as herein defined, in particular a conjugate of a CD44v6 specific antibody molecule and a maytansinoid. Preferably, the chemotherapeutic agent is a taxane, an epothilone, a vinca alkaloid, or another tubulin antagonist, a platinum compound, a camptothecin, a cryptophycin, a dolastatin, an epipodophyllotoxin, an alkylating agent, an

30

purine antagonist, a pyrimidine antagonist, or a DNA intercalator. In preferred embodiments, the chemotherapeutic agent is docetaxel, paclitaxel, RPR-116258A, epothilone A, B, C, D, E, or F, BMS-247550, BMS-310705, vinblastine, vindesine, vincristine, vinorelbine, combretastatin A4-phosphate, hydroxphenastatin, AVE 8062, 5 spongistatin 1, 2, 3, 4, 5, 6, 7, 8, or 9, E-7010, dolastatin, cemadotin hydrochloride, mivobulin isethionate, cryptophycin, camptothecin, topotecan, irinotecan, 9-aminocamptothecin, cisplatin, carboplatin, oxaliplatin, iproplatin, ormaplatin, tetraplatin, etoposide, teniposide, doxorubicin, daunorubicin, dactinomycin, plicamycin, mitomycin, bleomycin, idarubicin, cyclophosphamide, mechlorethamine, melphalan, chlorambucil, 10 procarbazine, dacarbazine, altretamine, carmustine, lomustine, semustine, methotrexate, mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, cytarabine, or azacytidine.

In a further aspect, the present invention relates to a method of treatment of cancer, 15 wherein an effective amount of a chemotherapeutic agent, as defined herein, is administered to a patient in need thereof in combination with a with a compound of Formula A(LB)<sub>n</sub>, as herein defined, in particular a conjugate of a CD44v6 specific antibody molecule and a maytansinoid.

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## Examples

### 1. Manufacturing and characterisation of BIWI 1

#### 5 1.1. Manufacturing of BIWI 1

Humanised recombinant antibodies BIWA 4 and BIWA 8 which have binding specificity for an epitope within SEQ ID NO: 1 were linked to the maytansinoid DM1 as described below. The conjugate of BIWA 4 with DM1 was designated BIWI 1.

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**Generation of stably transfected cell lines.** The genes coding for the light and heavy chains of BIWA 4, SEQ ID NO: 5 and SEQ ID NO: 7, were ligated into expression vector pAD-CMV1 (WO92/01055; NCBI GenBank Accession No. A32111) or pAD-CMV19 (NCBI GenBank Accession No. A32110). In the second antibody BIWA 8, the light chain  
15 was coded by a gene having SEQ ID NO: 9, while the heavy chain was the same as in BIWA 4. Stably transfected cell lines were generated by electroporation as follows. CHO DUX/57ss (dhfr negative mutant of Chinese Hamster Ovary cells, adapted for serum free suspension culture) were used. After trypsinisation and inactivation of trypsin with RPMI-10 (90% RPMI 1640, 10% heat inactivated fetal calf serum), cells were washed once with  
20 RPMI-0 (RPMI 1640 without serum), and  $1 \times 10^7$  cells were resuspended in 0.8 ml RPMI-0. After addition of the linearised DNA (20 µg per plasmid; cotransfection of vectors coding for light and heavy chain) the cells were electroporated using a Hoefer Electroporator under the following conditions: 1080 µF, 320 V, 1000 msec, 1 pulse. Cells were allowed to stand for 5 min, and were then diluted to 12500 cells/ml and 2500 cells/ml in alfa-MEM  
25 10d (90% MEM alfa without ribonucleosides and without desoxyribonucleosides (GIBCO BRL), 10% heat inactivated dialysed fetal calf serum). The cells were seeded into 96 well microtiter plates (200 µl/well, corresponding to 2500 and 500 cells/well respectively). Clones appeared after 10 days. Only the plates with 500 cells/well were followed up (3-6 clones/well). After 14-15 days, supernatants from each well were tested in a  $\kappa/\gamma$  ELISA. 53  
30 clones were seeded in 12 well plates in alfa-MEM 10d. After 3-6 days (depending on the confluency of the cells) supernatants were tested again in the  $\kappa/\gamma$  ELISA (serial dilutions) and quantitated using a human IgG1 standard. Cells were frozen and stored in liquid nitrogen. IgG contents of the 53 clones ranged from 12 - 417 ng/ml. 10 clones with the



highest expression level were selected and subcloned as follows: Cells of each clone were seeded into 96 well microtiter plates with densities of 1 and 5 cells/well in 100µl/well alpha-MEM 10d (1 plate for each clone and each density). 8 days later supernatants were diluted 1:2 and 100 µl of this dilution tested in the κ/γ ELISA and quantitated using a BIWA 4 preparation as standard. 5 subclones of each clone were transferred to 12 well plates. The IgG content ranged from 1.3 - 908 ng/ml. 14 clones with the highest expression level (384 - 908 ng/ml) were used for amplification with methotrexate as follows: Clones were initially cultured in 25 cm<sup>2</sup> flasks containing alpha-MEM 10d with 20, 50 and 100 nM methotrexate. After the outgrowth of clones the supernatants were tested in the κ/γ ELISA. In subsequent rounds of amplification the methotrexate concentration was raised up to 2000 nM. Initially the highest expression level ranged from 10.5-14.8 µg/ml (clone A31/100, 100 nM methotrexate). Further amplification with a methotrexate concentration of 500 nM gave an expression of 19-20 µg/ml (A31/500).

**Purification of antibody.** Antibody was purified from cell culture supernatant as follows. Antibody containing tissue culture supernatant was applied onto a 5 ml protein A sepharose column with a flow rate of 80-90 ml/h at 4 °C. After washing with 50 ml binding buffer (0.1 M sodium phosphate pH 7.5), the Ig fraction was eluted with elution buffer (0.1 M glycine-HCl pH 2.7). Absorption at 280 nm was monitored.

**Modification of BIWA 4 with SPP to form BIWA 4-SS-Py.** BIWA 4 was supplied in liquid form at a concentration of 5 mg/mL in a PBS formulation containing Tween 20. Prior to coupling of DM1 to the MAb the Tween 20 was removed. The MAb solution (40 mL) was diluted 15-fold with 25 mM MES buffer, pH 5.6, containing 50 mM NaCl (MES buffer) and then loaded onto a column (12.5 mL) of Sepharose S equilibrated in MES buffer (flow rate: 100 cm/hr). The column was washed with 10 column volumes of MES buffer. The antibody was eluted with MES buffer containing 400 mM NaCl. The antibody solution was dialysed against 50 mM potassium phosphate buffer, pH 6.5 containing 50 mM NaCl and 2 mM EDTA (Buffer A). The BIWA 4 antibody was modified using SPP ((2-Pyridyl)-5-dithiopentanoic acid N-hydroxy succinimid ester) to introduce dithiopyridyl groups. The MAb in Buffer A (185 mg, 8 mg/mL) was modified with a 7-fold molar excess of SPP in EtOH (5% v/v of MAb solution). The reaction proceeded for 90 minutes at ambient temperature. The reaction mixture was then subjected to gel filtration

chromatography through Sephadex G25F (2.6 x 31.5 cm column, 167 mL) equilibrated in Buffer A. MAb-containing fractions were pooled and the degree of modification was determined by measuring the absorbance at 280 nm and the change in absorbance at 343 nm caused by the release of 2-mercaptopyridine by the addition of DTT. The concentration of released 2-mercaptopyridine was calculated using an  $\epsilon_{343 \text{ nm}}$  of 8080  $\text{M}^{-1}\text{cm}^{-1}$ , and the concentration of MAb was calculated using an  $\epsilon_{280 \text{ nm}}$  of 224,000  $\text{M}^{-1}\text{cm}^{-1}$  after the absorbance at 280 nm has been corrected for the contribution from 2-mercaptopyridine. (2-mercaptopyridine  $A_{280 \text{ nm}} = A_{343 \text{ nm}} \times 5100/8080$ ). Recovery of the MAb was 99.6% with 5.5 releasable 2-mercaptopyridine groups linked per MAb molecule.

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**Conjugation of BIWA 4-SS-Py with DM1.** The above modified MAb (184 mg) in Buffer A was conjugated at 2.5 mg MAb/mL using a 1.7-fold molar excess of DM1 over releasable 2-mercaptopyridine groups. DM1 was added in DMA (3% v/v of MAb solution) and the reaction mixture was incubated at ambient temperature for 29 hours. The conjugate was then isolated by gel filtration chromatography on a column of Sephacryl S300 HR equilibrated in PBS (5 x 50 cm column, 980 mL, flow rate of 10 cm/hr). The conjugate eluted as a single peak at the position of monomeric MAb with a small amount of protein eluting earlier. Fractions were assayed for the number of DM1 molecules linked per MAb molecule. (Linked DM1 molecules were determined by measuring the absorbance at both 252 nm and 280 nm). Based on the results, fractions representing 63-77% of the column volume were pooled. The DM1/MAb ratio in the pooled solution was found to be 3.1 and the yield of conjugated BIWA 4 was 75% based on starting MAb. The conjugate, BIWI 1, was evaluated by SDS-PAGE performed under non-reducing conditions and found to be composed primarily of a monomer species (>95%) with a minor amount (<5%) of dimeric conjugate.

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### 1.2. Analysis of *in vitro* binding of BIWI 1.

The binding of BIWA 4 antibody and BIWI 1 conjugate to antigen-positive FaDu cells was determined. Cells ( $1-2 \times 10^5$ ) were incubated in 96-well plates with varying concentrations of antibody or conjugate on ice for 1 hour. The test article was washed from the plate and FITC-labeled anti-human IgG was added and the incubation on ice was continued in the

30

dark for 1 hour. After washing, the cells were fixed with 1% paraformaldehyde and analyzed on a fluorescence activated cell sorter (FACS). BIWA 4 antibody binds with an apparent  $K_D$  of  $1 \times 10^{-9}$  M and BIWI 1 binds with an apparent  $K_D$  of  $1.8 \times 10^{-9}$  M. Thus, conjugation with DM1 alters the binding affinity of the antibody only slightly if at all.

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### 1.3. *In vitro* cytotoxicity of BIWI 1

For determination of viable cells the Cell Titer 96<sup>®</sup> AQueous non-radioactive cell proliferation assay (Promega) was used. 5000 cells per well were seeded into 96-well plates in 90  $\mu$ l medium without phenole red. Cells were allowed to settle down for 1 to 3 h and then serial dilutions of the immunoconjugate in 10  $\mu$ l PBS were added. Cells without immunoconjugate served as negative control. Cells were incubated for 4 days at 37°C in a humified 5% CO<sub>2</sub> atmosphere and then 20  $\mu$ l MTS/ PMS were added according to the  
10 proliferation assay (Promega) was used. 5000 cells per well were seeded into 96-well plates in 90  $\mu$ l medium without phenole red. Cells were allowed to settle down for 1 to 3 h and then serial dilutions of the immunoconjugate in 10  $\mu$ l PBS were added. Cells without immunoconjugate served as negative control. Cells were incubated for 4 days at 37°C in a humified 5% CO<sub>2</sub> atmosphere and then 20  $\mu$ l MTS/ PMS were added according to the  
15 manufacturer's recommendation. After additional 1 to 4 h incubation at 37°C the absorbance at 490 nm was recorded using an ELISA plate reader. For each cell line triplicates were analyzed. The percentage of the surviving cell fraction and the IC<sub>50</sub> values were calculated using the GraphPad Prism<sup>®</sup> (Version 3.0) software.

The *in vitro* cytotoxicity of BIWI 1 was evaluated using the antigen-positive cell lines A431 and FaDu, and the antigen-negative cell line A459. Cells were exposed to different concentrations of BIWI 1 for 4 days, then stained with MTS/ PMS and assayed on an ELISA plate reader. The surviving fractions of cells were then calculated using the GraphPad Prism<sup>®</sup> software package. The results are shown in Figure 1. BIWI 1 was  
20 effective in killing the antigen-positive A431 cells with an IC<sub>50</sub> of about  $7.6 \times 10^{-8}$  M and the second antigen-positive cell line, FaDu, with an IC<sub>50</sub> of about  $2.4 \times 10^{-8}$  M. The antigen-negative cell line, A549, was effected by the conjugate with a surviving fraction of 50% at the highest concentration of BIWI 1 tested ( $5 \times 10^{-7}$  M). These results show that BIWI 1 is only slightly more cytotoxic against antigen-positive cells than antigen-negative  
25 cells *in vitro*. For comparison, another DM1-antibody conjugate has been shown to be at least 1000fold more cytotoxic against antigen-positive cell as compared to antigen-negative cells (Chari *et al.*, 1992).

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## 2. Efficacy studies in nude mice

### 2. 1. Xenograft models

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*In vivo* anti-tumor efficacy of BIWI 1 was tested in three nude mouse xenograft models applying antigen-positive human tumors: A431 (ATCC # CRL 1555; epidermoid carcinoma of the vulva), FaDu (ATCC # HTB 43; squamous cell carcinoma of the pharynx), and MDA-MB 453 (ATCC # HTB-131; breast carcinoma). The cells were received from ATCC and cultured in RPMI1640 medium containing 10% fetal calf serum and supplements.  $1 \times 10^6$  tumors cells were transplanted subcutaneously into the right flank of 6 week old female NMRI-nu/nu mice. Tumor growth was monitored by measuring tumor size. A tumor response was rated as complete response when the tumor completely disappeared at any time after start of treatment. The response was rated as partial response when the tumor volume decreased after treatment but thereafter started regrowing. The tolerability of the treatment was monitored by measuring mouse weight during the observation period.

### 20 2.2. BIWI 1 monotherapy in A431 xenografted nude mice

Mice were randomised into the following treatment groups (treatment/initial mean tumour volume/tumour volume range/number of mice):

- Group 1: Control (PBS) /  $185 \pm 217 \text{ mm}^3$  / 19 – 424  $\text{mm}^3$  / 5 mice.
- 25 Group 2: BIWA 4 (21 mg/kg/d) /  $133 \pm 115 \text{ mm}^3$  / 42 – 302  $\text{mm}^3$  / 5 mice.
- Group 3: BIWI 1 (2.1 mg/kg/d) /  $107 \pm 63 \text{ mm}^3$  / 42 – 205  $\text{mm}^3$  / 5 mice.
- Group 4: BIWI 1 (7 mg/kg/d) /  $132 \pm 73 \text{ mm}^3$  / 42 – 205  $\text{mm}^3$  / 5 mice.
- Group 5: BIWI 1 (21 mg/kg/d) /  $107 \pm 63 \text{ mm}^3$  / 42 – 205  $\text{mm}^3$  / 5 mice.

30 Groups of 5 mice were treated with 2.1 mg/kg/d BIWI 1, 7 mg/kg/d BIWI 1, 21 mg/kg/d BIWI 1, and 21 mg/kg/d control antibody, respectively. Treatment consisted of i.v. injections of BIWI 1 given on five consecutive days, starting at day 1. The average tumor volume of each group during the observation period is shown in Figure 2. Tumors treated

with control antibody showed similar growth as untreated tumors, the tumor volume doubling time was approximately 5 days. In animals treated either with 7 mg/kg/d BIWI 1 or 21 mg/kg/d BIWI 1 all tumors responded completely and disappeared around day 17. No tumor regrowth was observed until the end of the observation period (day 134).

- 5 Tumors treated with 2.1 mg/kg/d responded completely in 3/5 cases with no tumor regrowth until day 134. The remaining 2 tumors showed a partial response but ultimately regrew. These results show that BIWI 1 induces a dose-dependent anti-tumor response in A431 xenografted nude mice, with complete and long-lasting responses from 2.1 mg/kg/d BIWI 1 to 21 mg/kg/d BIWI 1. Unconjugated control antibody shows no anti-tumor effect.

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See Figure 2.

### 2.3. BIWI 1 monotherapy in FaDu xenografted nude mice

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Mice were randomised into the following treatment groups (treatment/initial mean tumour volume/tumour volume range/number of mice):

- Group 1: Control (PBS) /  $142 \pm 82 \text{ mm}^3$  / 34 – 268  $\text{mm}^3$  / 8 mice.  
Group 2: BIWA 4 (21 mg/kg/d) /  $134 \pm 86 \text{ mm}^3$  / 42 – 268  $\text{mm}^3$  / 6 mice.  
20 Group 3: BIWI 1 (2.1 mg/kg/d) /  $149 \pm 96 \text{ mm}^3$  / 50 – 268  $\text{mm}^3$  / 6 mice.  
Group 4: BIWI 1 (7 mg/kg/d) /  $132 \pm 97 \text{ mm}^3$  / 42 – 268  $\text{mm}^3$  / 6 mice.  
Group 5: BIWI 1 (21 mg/kg/d) /  $129 \pm 74 \text{ mm}^3$  / 50 – 231  $\text{mm}^3$  / 6 mice.

- Groups of 6 mice were treated with 2.1 mg/kg/d BIWI 1, 7 mg/kg/d BIWI 1, 21 mg/kg/d BIWI 1, and 21 mg/kg/d control antibody, respectively. Treatment consisted of i.v. injections of BIWI 1 given on five consecutive days, starting at day 1. The average tumor volume of each group during the observation period is shown in Figure 3. Tumors treated with control antibody and 2.1 mg/kg/d BIWI 1 showed similar growth as untreated tumors, the tumor volume doubling time was approximately 5 days. In animals treated with 21 mg/kg/d BIWI 1 all tumors responded completely and disappeared around day 24. No tumor regrowth was observed until the end of the observation period (day 107). 1/6 tumors treated with 7 mg/kg/d BIWI 1 responded completely, 3/6 tumors showed partial responses. The remaining 2 tumors grew similar to untreated control tumors. These results
- 25  
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show that BIWI 1 induces a dose-dependent anti-tumor response in FaDu xenografted nude mice, with complete and long-lasting responses from 7 mg/kg/d BIWI 1 to 21 mg/kg/d BIWI 1. Unconjugated control antibody shows no anti-tumor effect.

5 See Figure 3.

#### 2.4. BIWI 1 monotherapy in MDA-MB 453 xenografted mice

10 Groups of 6 mice were treated with 6.25 mg/kg BIWI 1, 12.5 mg/kg BIWI 1, and 25 mg/kg BIWI 1, respectively. Treatment consisted of i.v. injections of BIWI 1 given weekly for four weeks. The average tumor size at start of treatment was  $246 \pm 79 \text{ mm}^3$  (PBS),  $216 \pm 85 \text{ mm}^3$  (6.25 mg/kg BIWI 1),  $188 \pm 79 \text{ mm}^3$  (12.5 mg/kg BIWI 1), and  $207 \pm 96 \text{ mm}^3$  (25 mg/kg BIWI 1), respectively. The average tumor volume of each group during the  
15 observation period is shown in Figure 4. The initial tumor volume doubling time of the control tumors was approximately 5 days. In animals treated with 25 mg/kg BIWI 1 all tumors responded completely and disappeared around day 22 after start of treatment. No tumor regrowth was observed until the end of the observation period (day 64). Tumors treated with 12.5 mg/kg or 6.25 mg/kg responded completely in 5/6 cases in each dose  
20 group, and 4 animals of each group stayed tumor free until the end of the experiment. These results show that BIWI 1 induces anti-tumor responses in MDA-MB 453 xenografted nude mice when given once a week over a period of four weeks, with complete and long-lasting responses from 6.25 mg/kg BIWI 1 to 25 mg/kg BIWI 1.

25 See Figure 4.

#### 2.5. Tolerability of BIWI 1 monotherapy in nude mice

30 The tolerability of BIWI 1 monotherapy was determined by monitoring mouse weight during the whole duration of the experiment in the 2 models. The maximum observed average weight loss per group was 6% in FaDu xenografted mice treated with 21 mg/kg/d BIWI 1 (Figure 5). The weight loss started around day 3 of treatment and lasted until day

10, thereafter animals regained weight and behaved similar as control animals. In all other dose groups weight loss was similar to vehicle control (PBS). An average weight loss of 6% or less in all treatment groups indicates good tolerability of BIWI 1 treatment at the given doses in nude mice. As BIWI 1 does not cross-react with mouse CD44v6, only  
5 antigen-independent effects such as toxicity caused by free DM1 can be monitored in this experiment.

See Figure 5.

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## 2.6. BIWI 1 combination therapy with paclitaxel in the FaDu xenograft model

Mice were randomised into the following treatment groups (treatment/initial mean tumor volume/tumor volume range/number of mice):

- 15 Group 1: Control (PBS)/ 197 mm<sup>3</sup> / 65 – 402 mm<sup>3</sup> / 8 mice  
Group 2: Paclitaxel / 182 mm<sup>3</sup> / 65 – 302 mm<sup>3</sup> / 8 mice  
Group 3: BIWI 1 / 208 mm<sup>3</sup> / 65 – 382 mm<sup>3</sup> / 8 mice  
Group 4: BIWI 1 + paclitaxel/ 159 mm<sup>3</sup> / 79 – 335 mm<sup>3</sup> / 7 mice

20 Treatment consisted either of five i.v. injections of BIWI 1 (7 mg/kg corresponding to 100 µg/kg DM1) at day 1, 3, 5, 8, 10, or of six i.p. injections of paclitaxel (10 mg/kg) at day 2, 4, 6, 9, 11, 13, respectively, or a combination thereof. Control animals were treated with PBS.

25 The results of the experiments are summarised in Table 1. The mean relative tumor volumes during treatment are shown in Figure 6, individual tumor volumes are shown in Figure 7. Monotherapy with paclitaxel resulted in a tumor growth delay compared to PBS treated animals. BIWI 1 monotherapy resulted in a partial response in 3/ 8 animals which showed a clear delay in tumor growth compared to PBS-treated animals. In the  
30 combination group all tumors responded to treatment and showed clearly delayed tumor growth compared to control tumors. One tumor completely disappeared during treatment and the animal remained tumor free until the end of the observation period. These experiments demonstrate a markedly increased anti-tumor efficacy of a combination of

BIWI 1 with paclitaxel compared to the respective monotherapies, indicating a synergistic effect of the combination therapy.

**Table 1:** Initial tumour doubling time, growth delay factors and number of complete tumor regressions for different treatment groups

Treatment Group	Initial Tumour Doubling Time (d)	Growth Delay Factor	Complete Regression
Control	4	n.a.	0/8
Paclitaxel	7	0.75	0/8
BIWI 1	7	0.75	0/8
Combination BIWI 1 + Paclitaxel	39	8.75	1/7

See also Figures 6 and 7.

The tolerability of the treatment was determined by monitoring mouse weight during the whole duration of the experiment. The maximum observed average weight loss per group was less than 5% in in the combination group. In all other dose groups basically no weight loss upon treatment was observed. An average weight loss of less than 5% indicates good tolerability of the treatment at the given doses in nude mice.

## 2.7. BIWI 1 combination therapy with docetaxel and cisplatin in the FaDu xenograft model

Experiments were performed using nude mice xenografted subcutaneously with the human head and neck squamous cell carcinoma FaDu. To determine the efficacy of BIWI 1 as single drug given i.v., different dose levels were tested in a once weekly x 4 schedule. To assess possible antagonistic, additive or supraadditive effects in combination with chemotherapy, non-curative doses of BIWI 1 were combined with non-curative doses of docetaxel or cisplatin. The efficacy of these combinations was compared to those of the respective monotherapies by i) analysing the tumour volumes over time and ii) by determining the time for tumours to reach a certain size (tumour growth delay).

BIWI 1 (2.95 mg/ml protein, 53.6 µg/ml DM1, 3.7 molecules DM1/molecule Ab) was diluted in PBS according to the indicated DM1 concentrations. The dilution was stored at



+4°C during the whole treatment period.. The antibody solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse. In the control groups without BIWI 1 treatment animals were injected i.v. with PBS. Docetaxel (Taxotere<sup>®</sup>, Aventis, 10 mg/ml) was diluted in 0.9% NaCl according to the indicated concentrations. The solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse. Cisplatin (Cisplatin "Ebewe", 1 mg/ml) was diluted in 0.9% NaCl according to the indicated concentrations. The solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse

Tumour sizes and body weights were recorded 2-3 times per week. Tumour sizes were measured with a calliper (length and width) and the volume was calculated according to the following formula:  $\text{volume} = (\text{length}) \times (\text{width})^2 \times (\pi / 6)$ . The tolerability of the treatment was monitored by measuring mouse weight during the whole observation period. Animals were sacrificed when tumours reached a volume of more than 1600 mm<sup>3</sup> or when animals showed a weight loss of more than 15%. For calculation of the mean tumour volumes per group, the last values of tumour size for individual animals were carried forward in case that these animals had to be sacrificed due to large tumour burden before the whole group was terminated. In addition to the absolute tumour volume in mm<sup>3</sup>, the relative tumour volumes (RTV) were calculated as follows:

$$\text{RTV} = (\text{tumour volume at day } x) / (\text{tumour volume at start of treatment}) \times 100$$

For the evaluation of treatment efficacy the following parameters were calculated:

- T/C (%) = (mean RTV of treated group) / (mean RTV of control group) x 100

- T4T (tumour quadrupling time) = time until the mean RTV reaches 400%

- GDF<sub>4</sub> (growth delay factor) = T4T treated / T4T control.

In addition, for some experiments, the mean time for tumours to reach 8 times (T8T) their initial sizes were also calculated. The growth delay factor (GDF<sub>8</sub>) was then calculated accordingly. Percentages of mean relative tumour volumes of treated versus control groups (T/C) were calculated on the days indicated. Percentages of mean relative tumour volumes of treated versus control groups (T/C) were calculated on the days indicated. For statistical evaluation of combination therapies exact Wilcoxon tests were used. Tolerability was assessed by monitoring body weight changes.

To assess the efficacy of BIWI 1 in combination with the taxane docetaxel (Taxotere®), non-curative doses of both drugs were tested as single agents or in combination in nude mice xenografted with FaDu<sub>DD</sub> tumours. In pilot experiments a dose of 3 mg/kg docetaxel (q7dx4) was determined to be non-curative in this model. BIWI 1 was used at the non-  
5 curative dose of 200 µg/kg DM 1 and at a somewhat higher dose (300 µg/kg DM1).

Mice were randomised into the treatment groups and parameters for evaluating treatment efficacy are shown in Table 2. The control had a T4T of about 7 days. Monotherapy with docetaxel showed moderate efficacy in tumour growth retardation (T/C 56%, GDF<sub>4</sub>: 1.9).  
10 The effect was somewhat more pronounced in the two BIWI 1 monotherapy treatment groups with T/C 41-30% and GDF<sub>4</sub>: 2.8-4.4. 1/8 complete tumour regression could be observed with the higher BIWI 1 dose. In contrast, tumor growth was markedly inhibited in both combination treatments (T/C 3-2%), with none of the tumours reaching a relative tumour volume of 400% during the observation period of 95 days (GDF<sub>4</sub> > 13.3).  
15 Combination of docetaxel with the lower dose of BIWI 1 resulted in 7/8 complete tumour regressions, while in the combination group with the higher BIWI 1 dose all tumours disappeared. These results indicate a supraadditive effect of the combination treatments in comparison to the respective monotherapies. Treatment was well tolerated in all groups, except for a slight unexplained weight loss (approx. -5%) in the docetaxel monotherapy  
20 group.

**Table 2:** Combination of BIWI 1 with docetaxel in nude mice xenografted with FaDu<sub>DD</sub> tumours: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy (for definition of the parameters see section 4.4). Treatment schedule: once weekly for four weeks (q7dx4); 8 animals per group. The day for which T/C was evaluated is indicated.

Groups	Initial TV mean (mm <sup>3</sup> )	Initial TV range (mm <sup>3</sup> )	T/C (d 16)	T4T (d)	GDF <sub>4</sub>
Control (PBS)	166	113-268	100%	7.2	1.0
BIWI 1 200 µg/kg DM1	157	79-335	41%	20.1	2.8
BIWI 1 300 µg/kg DM1	183	65-382	30%	31.1	4.4
Docetaxel 3 mg/kg	193	79-302	56%	13.5	1.9
BIWI 1 200 µg/kg DM1 + docetaxel 3 mg/kg	160	92-268	3%	>95	>13.3
BIWI 1 300 µg/kg DM1 + docetaxel 3 mg/kg	200	113-424	2%	>95	>13.3

A different set of experiments was done with cisplatin. In a pilot experiment, a dose of 4 mg/kg cisplatin administered i.v. four times in weekly intervals was determined to be non-curative in this model, and also represents the maximum tolerated dose in the mouse strain used. This dose of cisplatin was combined with BIWI 1 at the non-curative doses of 200 µg/kg DM1 and 300 µg/kg DM1 already used in the previous experiment. Mice were randomised into the treatment groups and parameters for evaluating treatment efficacy are shown in Table 3. The control had a T4T of about 11 days. Monotherapy with BIWI 1 showed moderate efficacy at both dose levels (T/C 59-27%, GDF<sub>4</sub>: 2.2-2.3), while cisplatin as single agent was ineffective (T/C 100%, GDF<sub>4</sub>: 1.1). In contrast, combination of cisplatin with BIWI 1 showed a clear therapeutic advantage with GDF<sub>4</sub> of 4 in the group with the lower BIWI 1 dose and >6.8 in the group with the higher BIWI 1 dose. In the latter combination group 2/8 complete tumour regressions could be observed. These results indicate a supraadditive effect of the combination treatments in comparison to the respective monotherapies. Treatment was well tolerated in all groups.

**Table 3:** Combination of BIWI 1 with cisplatin in nude mice xenografted with FaDu<sub>pp</sub> tumours: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy. Treatment schedule: once weekly for four weeks (q7dx4); 8 animals per group. The day for which T/C was evaluated is indicated.

Groups	Initial TV mean (mm <sup>3</sup> )	Initial TV range (mm <sup>3</sup> )	T/C (d 19)	T4T (d)	GDF <sub>4</sub>
Control (PBS)	183	79-268	100%	10.7	1.0
BIWI 1 200 µg/kg DM1	237	113-382	59%	23.2	2.2
BIWI 1 300 µg/kg DM1	178	113-268	27%	24.8	2.3
Cisplatin 4 mg/kg	218	132-268	100%	11.3	1.1
BIWI 1 200 µg/kg DM1 + cisplatin 4 mg/kg	239	132-302	16%	42.7	4.0
BIWI 1 300 µg/kg DM1 + cisplatin 4 mg/kg	236	113-382	3%	>72.3	>6.8

## 2.8. BIWI 1 combination therapy with doxorubicin or docetaxel in a human breast carcinoma model xenograft model

Similar experiments as outlined under 2.7 were undertaken in a human breast carcinoma xenograft model. Experiments were performed using nude mice xenografted subcutaneously with the human breast carcinoma cell line MDA-MB-453. To determine the efficacy of BIWI 1 as single drug given i.v., different dose levels were tested in a once weekly x 4 schedule. To assess possible antagonistic, additive or supraadditive effects in combination with chemotherapy, non-curative doses of BIWI 1 were combined with non-curative doses of different cytostatics. The efficacy of these combinations was compared to those of the respective monotherapies by i) analysing the tumour volumes over time and ii) by determining the time for tumours to reach a certain size (tumour growth delay).

BIWI 1 (protein concentration 2-3 mg/ml, DM1 concentration 30-50 µg/ml, 3.3-3.7 molecules DM1 per molecule antibody) was diluted in PBS according to the indicated

DM1 concentrations. The dilutions were prepared freshly for each application or stored at +4°C during the whole treatment period. According to the amount of BIWI 1 needed 50-500 µl of the antibody solution were injected into the tail vein. BIWI 1 dose levels are indicated in µg DM1 per kg body weight (µg/kg DM1). Doxorubicin (Doxorubicin  
5 "Ebewe"®, 2 mg/ml) was diluted in PBS. 200 µl of solutions with different concentrations were injected into the tail vein. Docetaxel (Taxotere®, Aventis, 10 mg/ml) was diluted in 0.9% NaCl. 200 µl of solutions with different concentrations were injected into the tail vein. For combination therapies, BIWI 1 was injected approximately 4 hours prior to the cytostatic drug.

10

The xenograft model was handled slightly different as outlined under section 2.1. Briefly,  $1 \times 10^7$  MDA-MB-453 cells (ATCC No. HTB-131) were injected into the mammary fat pad of female NMRI nu/nu mice. After reaching a volume of approximately 1000 mm<sup>3</sup> tumours were excised, cut into 4 x 4 mm pieces and passaged further subcutaneously. Tumour  
15 fragments were cryoconserved in 90% RPMI 1640 / 10% dimethylsulfoxide (slow freezing process down to -80°C, storage in liquid nitrogen). For further passaging, frozen tumour fragments were thawed, washed 3 times in PBS and implanted subcutaneously into the right and left flanks of a 4-5 female NMRI nu/nu mice. Tumours of these mice were then used to prepare the animals for a therapy experiment. Tumours were excised with a size of  
20 approximately 10 mm in diameter, cut into 2 x 2 mm pieces and implanted subcutaneously into the right flank of 6-8 week old female NMRI nu/nu mice. Approximately twice as many animals as needed for the experiment were prepared. When tumours reached a size of 5-10 mm in diameter (10-14 days after implantation), animals were randomised and treatments were started. Animals were obtained from Harlan (Germany) or M&B  
25 (Denmark). Tumour sizes and body weights were recorded as described in section 2.7.

In pilot experiments, a dose of 6 mg/kg, doxorubicin (q7dx4) was determined to be non-curative in this model. Since it was possible to cure 4/6 of the tumour bearing animals with a BIWI 1 dose of 100 µg/kg DM1, q7dx4, lower doses of the antibody conjugate (75 and  
30 50 µg/kg DM1) were used in the combination experiment to assure non-curative treatment with BIWI 1 alone. Mice were randomised into the treatment groups and parameters for evaluating treatment efficacy are shown in Table 4. The control group, as well as the animals treated with a BIWI 1 dose of 50 µg/kg DM1 or 6 mg/kg doxorubicin had a T4T

of about 8 days. In the group treated with BIWI 1 at 75 µg/kg DM1 a T4T of 13 days was observed. Combination of BIWI 1 at a dose of 50 µg/kg DM1 with doxorubicin showed a small increase in efficacy over the respective monotherapies (T4T: 11 days). However when BIWI 1 at 75 µg/kg DM1 was combined with doxorubicin the T4T was 43 days, showing a supra-additive therapeutic advantage compared to the respective monotherapies (GDF<sub>4</sub> of 5.3 compared to no delay of the doxorubicin treatment and GDF<sub>4</sub> of 1.6 of the BIWI 1 (75 µg/kg DM1) monotherapy). Treatment was well tolerated in all groups. Only two animals in the doxorubicin monotherapy group and one animal in the combination group with 75 µg/kg DM1 showed transient weight loss of more than 12%.

**Table 4:** Combination of BIWI 1 with doxorubicin in nude mice xenografted with MDA-MB-453 tumours: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy (for definition of the parameters see above).

Treatment schedule: once weekly for four weeks (q7dx4); 6 animals per group. The day for which T/C was evaluated is indicated.

Groups	Initial TV mean (mm <sup>3</sup> )	Initial TV range (mm <sup>3</sup> )	T/C (d13)	T4T (d)	GDF <sub>4</sub>
Control (PBS)	244	151-424	100 %	8.0	1.0
BIWI 1 50 µg/kg DM1	261	180-302	68 %	8.0	1.0
BIWI 1 75 µg/kg DM1	272	205-382	59 %	13.0	1.6
Doxorubicin 6 mg/kg	249	132-424	66 %	7.8	1.0
BIWI 1 50 µg/kg DM1 + doxorubicin 6 mg/kg	274	180-424	65 %	10.7	1.3
BIWI 1 75 µg/kg DM1 + doxorubicin 6 mg/kg	293	180-424	29 %	42.6	5.3

In a second set of experiments, non-curative doses of docetaxel, which had been determined before, were combined with BIWI 1. Parameters for evaluating treatment efficacy are shown in Table 5. No difference between the control group and the animals treated with 3 mg/kg docetaxel could be seen during the whole treatment period (T4T 11-9 days). In the two other monotherapy groups (6 mg/kg docetaxel and BIWI 1) a moderate

tumour growth delay at the end of the treatment period could be observed (T/C: 75-71%, T4T of 14 days compared to a T4T of 11 days of the control, corresponding to a GDF<sub>4</sub> of 1.3). In contrast, both combination treatments showed an increased tumour growth retardation when compared to the respective monotherapies. BIWI 1 combined with 6 mg/kg docetaxel had a T4T of 37 days (T/C: 19%) compared to 14 days of the monotherapies. A similar effect could be observed when 3 mg/kg docetaxel was combined with BIWI 1 (T4T of 32 days, T/C: 30%). In both combination experiments, comparison of the growth delay factors of the combination treatments and the monotherapies suggests a supraadditive effect. BIWI 1 combined with 6 mg/kg docetaxel gave a GDF<sub>4</sub> of 3.3 compared to 1.3 of the respective monotherapies. In the other combination group with 3 mg/kg docetaxel (GDF<sub>4</sub> of 2.9) the monotherapy with docetaxel was by itself ineffective (GDF<sub>4</sub>: 0.8) and treatment with BIWI 1 alone gave a GDF<sub>4</sub> of 1.3. Treatment was well tolerated in all groups.

**Table 5:** Combination of BIWI 1 with docetaxel in nude mice xenografted with MDA-MB-453 tumours: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy.

Treatment schedule: once weekly for four weeks (q7dx4); 8 animals per group. The day for which T/C was evaluated is indicated.

Groups	Initial TV mean (mm <sup>3</sup> )	Initial TV range (mm <sup>3</sup> )	T/C (d16)	T4T (d)	GDF <sub>4</sub>
Control (0.9% NaCl)	239	179-329	100 %	11.0	1.0
BIWI 1 75 µg/kg DM1	235	147-299	75 %	14.1	1.3
Docetaxel 6 mg/kg	253	179-306	71 %	13.9	1.3
Docetaxel 3 mg/kg	288	122-586	102 %	9.0	0.8
BIWI 1 75 µg/kg DM1 + docetaxel 6 mg/kg	327	169-486	19 %	36.8	3.3
BIWI 1 75 µg/kg DM1 + docetaxel 3 mg/kg	280	192-377	30 %	31.7	2.9

## **2.9 BIWI 1 combination therapy with capecitabine in a human breast carcinoma model xenograft model**

Similar experiments as outlined under 2.7 were undertaken in a human breast carcinoma  
5 xenograft model, wherein BIWI 1 therapy was combined with the 5'-deoxy-5-fluorouridine  
prodrug capecitabine (Xeloda<sup>®</sup>). The xenograft model MDA-MB-453 was handled as in  
section 2.8.

BIWI 1 (protein concentration 2.95 mg/ml, 53.6 µg DM1/ml, 3.7 molecules  
10 DM1/molecule antibody) was diluted in PBS to a DM1 concentration of 75 µg/kg body  
weight. The dilution was stored at +4°C during the whole treatment period. The antibody  
solution was injected into the tail vein with an injection volume of 200 µl for a 25 g  
mouse. In the control groups without BIWI 1 treatment animals were injected i.v. with  
PBS. BIWI 1 was given i.v. into the tail vein at a dose of 75 µg DM1/kg once weekly, for  
15 three weeks. Capecitabine (150 mg tablets, Roche) was given intragastrically (by gavage  
needle, as a suspension in water) at a dose of 375 or 500 mg/kg during three weeks on 5  
consecutive days per week.

The efficacy of the combinations was compared to those of the respective monotherapies  
20 as outlined in section 2.8. To assess the efficacy of BIWI 1 in combination with  
capecitabine, non-curative doses of both drugs were tested as single agents or in  
combination in nude mice xenografted with MDA-MB-453 tumours. In previous  
experiments a BIWI 1 dose of 75 µg/kg DM1 was shown to be non-curative. For  
capecitabine, doses of 500 and 750 mg/kg, (q1dx5, 4 cycles) were determined to be non-  
25 curative, while a dose of 250 mg/kg was completely ineffective.

For the combinations experiments BIWI 1 with 75 µg/kg DM1 was combined with 375 or  
500 mg/kg capecitabine. Mice were randomised into the treatment groups shown in Table  
6. The control had a T4T of about 10 days. The different monotherapies showed moderate  
30 efficacy in tumour growth retardation with growth delay factors (GDF<sub>4</sub>) of 2.6 for BIWI 1,  
1.3 for the lower dose of capecitabine and 2.9 for the higher dose of capecitabine. No  
complete regressions were seen. When BIWI 1 was combined with 375 mg/kg or 500  
mg/kg capecitabine the growth delay factors increased to 4.5 and 5.3 respectively and T/C



at day 25 was 4% and 3% respectively. In both combination groups one complete regression could be observed.

**Table 6:** Combination of BIWI 1 with capecitabine in nude mice xenografted with MDA-MB-453 tumours: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy.

Treatment schedules:

BIWI 1: once weekly for three weeks (q7dx3); Capecitabine: daily 5 times per week for three weeks (q1d5x, 3 cycles).

8 animals per group. In the groups treated with 375 mg/kg capecitabine as monotherapy and in combination with BIWI 1 only 7 animals could be evaluated (death not related to therapy or tumour growth).

The day for which T/C was evaluated is indicated.

Groups	Initial TV mean (mm <sup>3</sup> )	Initial TV range (mm <sup>3</sup> )	T/C (d25)	T4T (d)	GDF <sub>4</sub>
Control (PBS/H <sub>2</sub> O)	139	84-180	100 %	10.3	1.0
BIWI 1 75 µg/kg DM1	132	58-212	31%	26.9	2.6
Capecitabine 500 mg/kg	130	78-173	23%	30.0	2.9
Capecitabine 375 mg/kg	130	82-227	58%	13.8	1.3
BIWI 1 75 µg/kg DM1 + Capecitabine 500 mg/kg	141	89-204	3%	54.7	5.3
BIWI 1 75 µg/kg DM1 + Capecitabine 375 mg/kg	133	80-233	4%	46.2	4.5

15

Combination of BIWI 1 at 75 µg/kg DM1 given once weekly for 3 weeks with capecitabine at 375 or 500 mg/kg given 5 times weekly for 3 weeks, showed a significant increase of the therapeutic effect from day 7 on, when comparing tumour sizes to those of the respective monotherapies. Comparison of the tumour growth delay factors indicates a synergistic effect of BIWI 1 and capecitabine in this combination setting. All of the treatments were well tolerated without any significant weight loss of the animals.

20

## 2.10 BIWI 1 combination therapy with paclitaxel in a human breast carcinoma model xenograft model

- 5 Similar experiments as outlined under 2.6 were undertaken in a human breast carcinoma xenograft model, wherein BIWI 1 therapy was combined with the taxane paclitaxel. BIWI was given i.v. into the tail vein at doses of 50  $\mu\text{g DM1/kg/day}$  or 100  $\mu\text{g DM1/kg/day}$  for five consecutive days. Paclitaxel (Taxol<sup>®</sup>) was given i.v. at 20 or 10 mg/kg, 4 hours after BIWI 1 treatment. In mice bearing breast carcinoma xenografts with a volume of
- 10 approximately 100 mm<sup>3</sup> BIWI 1 monotherapy was efficacious at a dose level of 100  $\mu\text{g DM1/kg}$ , showing complete regression of all (12/12) tumours. A dose level of 50  $\mu\text{g DM1/kg}$  resulted in 2/12 complete tumour regressions. When BIWI at 50  $\mu\text{g DM1/kg}$  was combined with paclitaxel at 20 or 10 mg/kg, a significant increase of the therapeutic effect was observed (Percentage of median relative tumour volumes of treated versus control
- 15 groups = T/C of 0% for 20 mg paclitaxel /kg and 9 % for 10 mg paclitaxel /kg, respectively) in comparison to the respective monotherapies (T/C 19 % for BIWI 1, 13 % for 20 mg paclitaxel/kg, and 50 % for 10 mg paclitaxel/kg). All treatments were well tolerated without any significant weight loss of the animals.

20

### Brief Description of Drawings

**Figure 1:** In vitro cytotoxicity of BIWI 1. The antigen-positive cell lines A431 and FaDu and the antigen-negative cell line A549 were used.

25

**Figure 2:** Efficacy of BIWI 1 treatment in nude mice xenografted with A431 tumors. The average tumor volumes per group with standard deviations are shown, the treatment groups are indicated. The arrow indicates start of treatment (day 1).

- 30 **Figure 3:** Efficacy of BIWI 1 treatment in nude mice xenografted with FaDu tumors. The average tumor volumes per group with standard deviations are shown, the treatment groups are indicated. The arrow indicates start of treatment (day 1).

**Figure 4:** Efficacy of BIWI 1 treatment in nude mice xenografted with MDA-MB 453 tumors. The average tumor volumes per group with standard deviations are shown, the treatment groups are indicated. The arrows indicate the treatment days.

- 5 **Figure 5:** Tolerability of BIWI 1 treatment. The average body weight change of all treatment groups in the 2 investigated models is shown. Day 1: start of treatment.

**Figure 6:** Efficacy of BIWI 1 combination treatment with paclitaxel in nude mice xenografted with FaDu tumors. The average tumor volume per group with standard deviations is shown.

- 10 **Figure 7:** Efficacy of BIWI 1 combination treatment with paclitaxel in nude mice xenografted with FaDu tumors. The individual tumor volumes per group are shown.

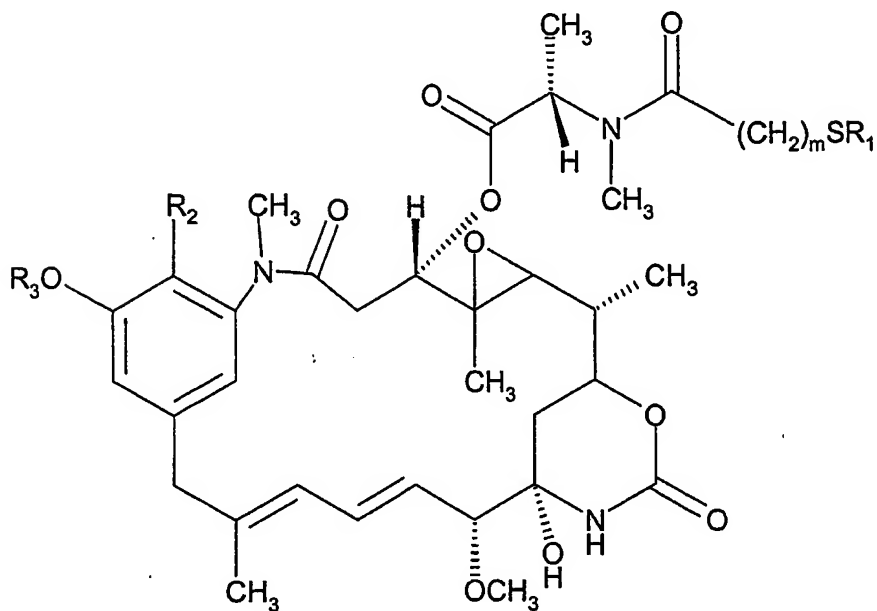
- Figure 8:** BIWI 1 combination treatment with paclitaxel in nude mice xenografted with FaDu tumors. Tolerability of treatment. The average body weight change of all treatment groups is shown. Day 1: start of treatment.
- 15

## Claims

1. Use of a compound of formula  
 $A(LB)_n$   
5 wherein  
A is an antibody molecule which is specific for CD44;  
L is a linker moiety;  
B is a compound which is toxic to cells; and  
n is a decimal number with  $n = 1$  to 10  
10  
for the preparation of a pharmaceutical composition for the treatment of cancer,  
wherein said compound is used or is for use in combination with a chemotherapeutic  
agent.
- 15 2. The use of claim 1 wherein said linker moiety has a chemical bond capable of being  
cleaved inside a cell.
3. The use of claim 2 wherein said chemical bond is a disulfide bond.
- 20 4. The use of claims 1 to 3, wherein the antibody molecule is specific for the exon v6 of  
human CD44.
5. The use of claims 1 to 4, wherein the antibody molecule is specific for an epitope  
within the amino acid sequence SEQ ID NO: 3.
- 25 6. The use of claims 1 to 5, wherein the antibody molecule is the monoclonal antibody  
VFF-18 (DSM ACC2174) or a recombinant antibody having the complementary  
determining regions (CDRs) of VFF-18.
- 30 7. The use of claims 1 to 6, wherein the antibody molecule comprises light chains having  
the amino acid sequence SEQ ID NO: 4, or SEQ ID NO: 8, and heavy chains having  
the amino acid sequence SEQ ID NO: 6.

8. The use of claims 1 to 7, wherein the toxic compound **B** is a maytansinoid.

9. The use of claim 8 wherein the maytansinoid has the formula



(Formula (II))

5

wherein

$R_1$  represents H or  $SR_4$ , wherein  $R_4$  represents methyl, ethyl, linear alkyl, branched alkyl, cyclic alkyl, simple or substituted aryl, or heterocyclic;

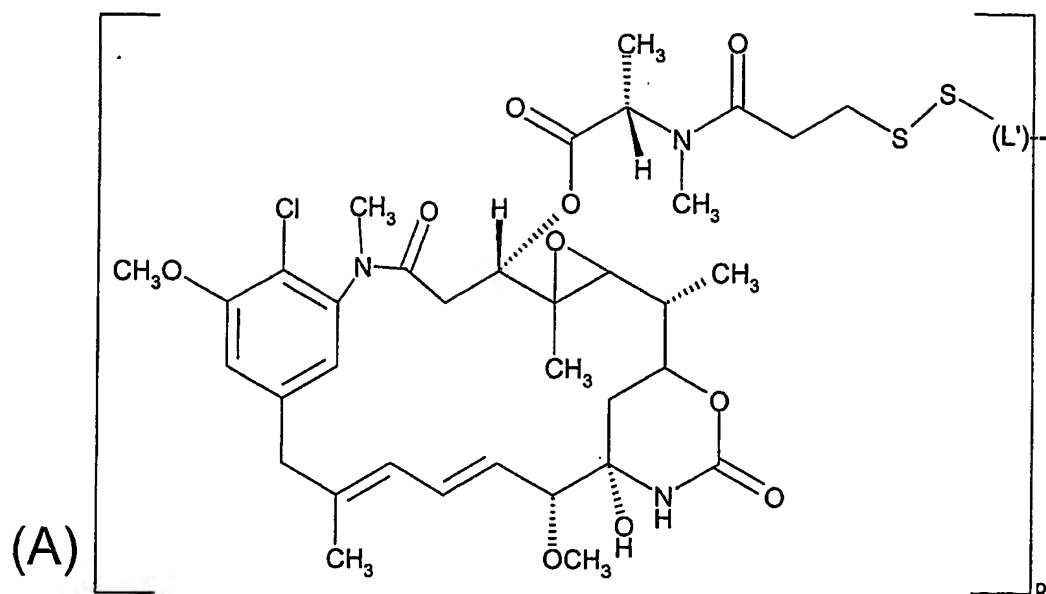
$R_2$  represents Cl or H;

10  $R_3$  represents H or  $CH_3$ ; and

$m$  represents 1, 2, or 3.

10. The use of claim 9, wherein  $R_1$  is H or  $CH_3$ ,  $R_2$  is Cl,  $R_3$  is  $CH_3$ , and  $m = 2$

15 11. The use of claims 1 to 10, wherein the compound **A(LB)<sub>n</sub>** is of formula



wherein

A is an antibody molecule which is specific for CD44,

(L') is an optional linker moiety.

p is a decimal number with  $p = 1$  to  $10n$

12. The use of claims 1 to 11 wherein p is 3 to 4.

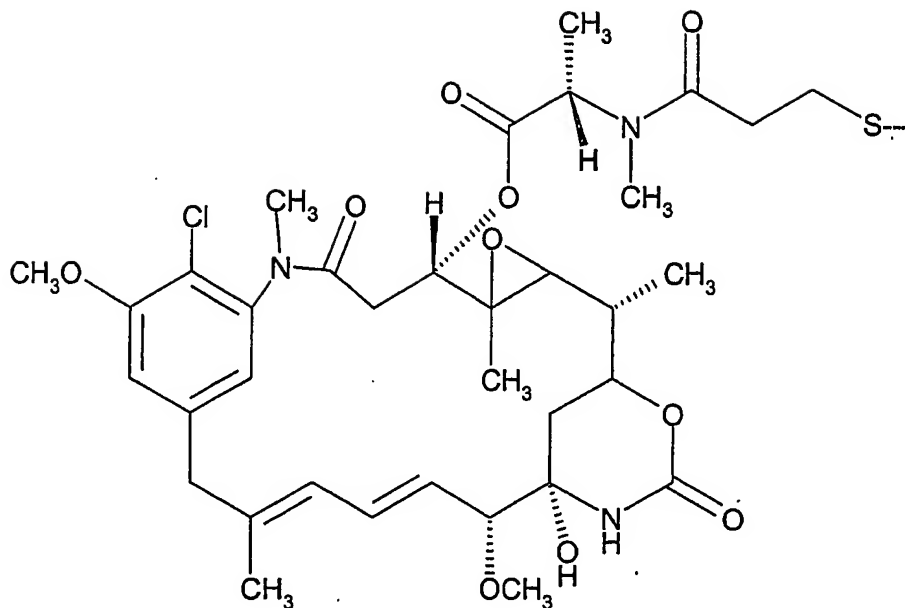
13. The use of any one of claims 1 to 12, wherein the chemotherapeutic agent is a tubulin  
5 binding agent.

14. The use of claims 13, wherein the chemotherapeutic agent is a microtubule stabilizing  
agent.

10 15. The use of claim 14, wherein the chemotherapeutic agent is a taxane or an epothilone.

16. The use of claim 15, wherein the chemotherapeutic agent is paclitaxel, docetaxel, RPR-  
116258A, epothilone A, B, C, D, E, or F, BMS-247550, or BMS-310705.

17. The use of claim 13, wherein the chemotherapeutic agent is a microtubule destabilizing agent.
18. The use of claim 17, wherein the chemotherapeutic agent is a vinca alkaloid.
- 5 19. The use of claim 18, wherein the chemotherapeutic agent is vinblastine, vincristine, vinflunine, vindesine, navelbine, or vinorelbine.
- 10 20. Use of a conjugate of a CD44v6 specific antibody molecule and a maytansinoid for the manufacture of a pharmaceutical composition for the treatment of cancer, wherein said conjugate is used or is for use in combination with a chemotherapeutic agent.
21. The use of of claim 20, wherein the antibody molecule is specific for an epitope within the amino acid sequence SEQ ID NO: 3.
- 15 22. The use of claim 21, wherein the antibody molecule is the monoclonal antibody VFF-18 (DSM ACC2174) or a recombinant antibody having the complementary determining regions (CDRs) of VFF-18.
- 20 23. The use of of any one of claims 20 to 22, wherein said antibody molecule comprises light chains having the amino acid sequence SEQ ID NO: 4, or SEQ ID NO: 8, and heavy chains having the amino acid sequence SEQ ID NO: 6.
- 25 24. The use of any one of claims 2013 to 2315, wherein the maytansinoid is linked to the antibody molecule by a disulfide moiety.
25. The use of any one of claims 20 to 24, wherein the maytansinoid has the formula

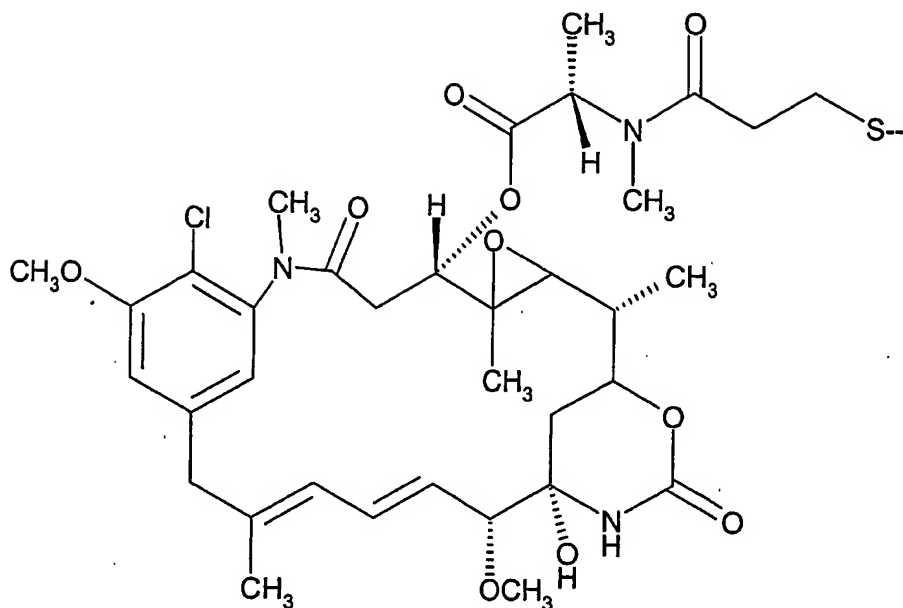


Formula (IV)

26. The use of a conjugate of a CD44v6 specific antibody molecule and a maytansinoid for the manufacture of a medicament for the treatment of cancer, wherein said compound is used or is for use in combination with a chemotherapeutic agent, and wherein the antibody comprises light chains having the amino acid sequence SEQ ID NO: 4, and heavy chains having the amino acid sequence SEQ ID NO: 6, and wherein the maytansinoid has the formula



56



(Formula IV)

and is linked to the antibody through a disulfide bond.

27. The use of any one of claims 20 to 26, wherein one or more maytansinoid residues are linked to an antibody molecule.
28. The use of claim 27, wherein 3 to 4 maytansinoid residues are linked to an antibody molecule.
29. The use of any one of claims 20 to 28, wherein the maytansinoid is linked to the antibody molecule through a  $-S-CH_2CH_2-CO-$ , a  $-S-CH_2CH_2CH_2CH_2-CO-$ , or a  $-S-CH(CH_3)CH_2CH_2-CO-$  group.
30. The use of any one of claims 20 to 29, wherein the chemotherapeutic agent is a tubulin binding agent.
31. The use of claims 30, wherein the chemotherapeutic agent is a microtubule stabilizing agent.
32. The use of claim 31, wherein the chemotherapeutic agent is a taxane or an epothilone.

33. The use of claim 32, wherein the chemotherapeutic agent is paclitaxel, docetaxel, RPR-116258A, BMS-247550, BMS-310705, or epothilone A, B, C, D, E, or F.
- 5 34. The use of claim 30, wherein the chemotherapeutic agent is a microtubule destabilizing agent.
35. The use of claim 34, wherein the chemotherapeutic agent is a vinca alkaloid.
- 10 36. The use of claim 35, wherein the chemotherapeutic agent is vinblastine, vincristine, vindesine, vinflunine, navelbine, or vinorelbine.
- 15 37. The use of any one of claims 1 to 36, wherein the cancer is head and neck squameous cell carcinoma, esophagus squameous cell carcinoma, lung squameous cell carcinoma, skin squameous cell carcinoma, cervix squameous cell carcinoma, breast adenocarcinoma, lung adenocarcinoma, pancreas adenocarcinoma, colon adenocarcinoma, or stomach adenocarcinoma.
- 20 38. The use of any one of claims 1 to 37, wherein said compound  $A(LB)_n$  or conjugate, and said chemotherapeutic agent are formulated in separate pharmaceutical compositions.
- 25 39. The use of any one of claims 1 to 37, wherein said compound  $A(LB)_n$  or conjugate and said chemotherapeutic agent are formulated in one single pharmaceutical composition.
- 30 40. Method of treatment of cancer in a patient in need thereof, comprising administering to the patient a therapeutically effective amount of a compound  $A(LB)_n$  as defined in any one of claims 1 to 12, or a conjugate as defined in any one of claims 20 to 29, in combination with a chemotherapeutic agent as defined in any one of claims 13 to 19, or 30 to 36.
41. The method of claim 40, wherein the cancer is head and neck squameous cell carcinoma, esophagus squameous cell carcinoma, lung squameous cell carcinoma, skin

squamous cell carcinoma, cervix squamous cell carcinoma, breast adenocarcinoma, lung adenocarcinoma, pancreas adenocarcinoma, colon adenocarcinoma, or stomach adenocarcinoma.

- 5 42. The method of claim 40 or 41, wherein the compound  $A(LB)_n$  or conjugate, and the chemotherapeutic agent are administered separately.
43. The method of claim 40 or 41, wherein the compound  $A(LB)_n$  or conjugate, and the chemotherapeutic agent are administered as components of a single pharmaceutical composition.
- 10 44. Pharmaceutical composition comprising a compound  $A(LB)_n$  as defined in any one of claims or according to claims 1 to 12, or a conjugate as defined in any one of claims 20 to 29, together with a chemotherapeutic agent as defined in any one of claims 13 to 19, or 30 to 36, and optionally further comprising one or more pharmaceutically acceptable carrier(s), diluent(s), or excipient(s).
- 15 45. A kit comprising, in separate pharmaceutical compositions, a compound  $A(LB)_n$  as defined in any one of claims 1 to 12, or a conjugate as defined in any one of claims 20 to 29, and a chemotherapeutic agent as defined in any one of claims 13 to 19, or 30 to 36.
- 20 46. Use of a chemotherapeutic agent for the preparation of a pharmaceutical composition for the treatment of cancer, wherein said chemotherapeutic agent is used or is for use in combination with a compound of Formula  $A(LB)_n$  as defined in any of the preceding claims.
- 25 47. Use of a chemotherapeutic agent for the preparation of a pharmaceutical composition for the treatment of cancer, wherein said chemotherapeutic agent is used or is for use in combination with a conjugate as defined in any one of claims 20 to 29.
- 30 48. The use of claim 46 or 47, wherein the chemotherapeutic agent is a taxane, an epothilone, a vinca alkaloid, a platinum compound, a camptothecin, a cryptophycin, a

dolastatin, a 5,6-dihydroindolo[2,1-a]isoquinoline derivative, a spongistatin, an epipodophyllotoxin, an alkylating agent, an purine antagonist, a pyrimidine antagonist, or a DNA intercalator.

- 5 49. The use of claim 48, wherein the chemotherapeutic agent is docetaxel, paclitaxel, RPR-116258A, epothilone A, B, C, D, E, or F, BMS-247550, BMS-310705, vinblastine, vindesine, vincristine, vinorelbine, vinflunine, navelbine, combretastatin A4-phosphate, hydroxphenastatin, AVE 8062, spongistatin 1, 2, 3, 4, 5, 6, 7, 8, or 9, E-7010, dolastatin, cemadotin hydrochloride, mivobulin isethionate, cryptophycin,
- 10 camptothecin, topotecan, irinotecan, 9-aminocamptothecin, cisplatin, carboplatin, oxaliplatin, iproplatin, ormaplatin, tetraplatin, etoposide, teniposide, doxorubicin, daunorubicin, dactinomycin, plicamycin, mitomycin, bleomycin, idarubicin, cyclophosphamide, mechlorethamine, melphalan, chlorambucil, procarbazine, dacarbazine, altretamine, carmustine, lomustine, semustine, methotrexate,
- 15 mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, capecitabine, cytarabine, or azacytidine.

1/8

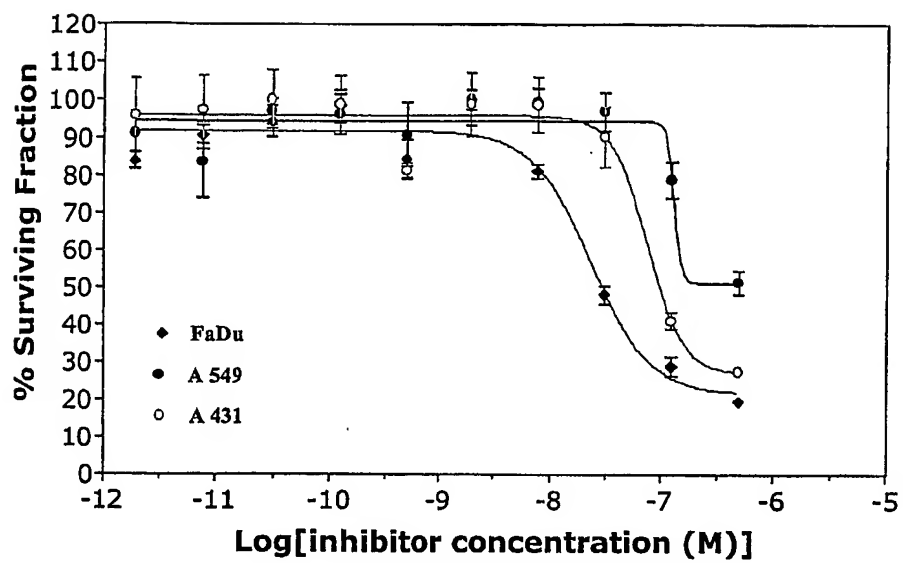
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Figure 1

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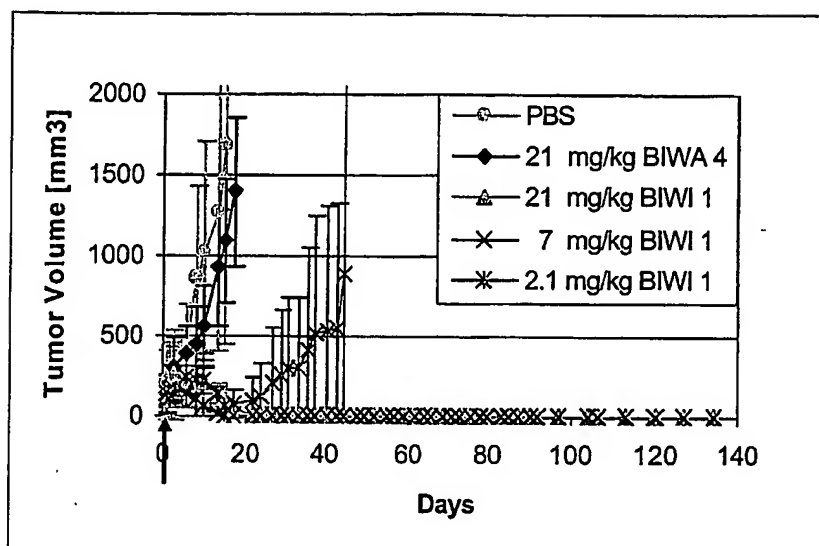


Figure 2

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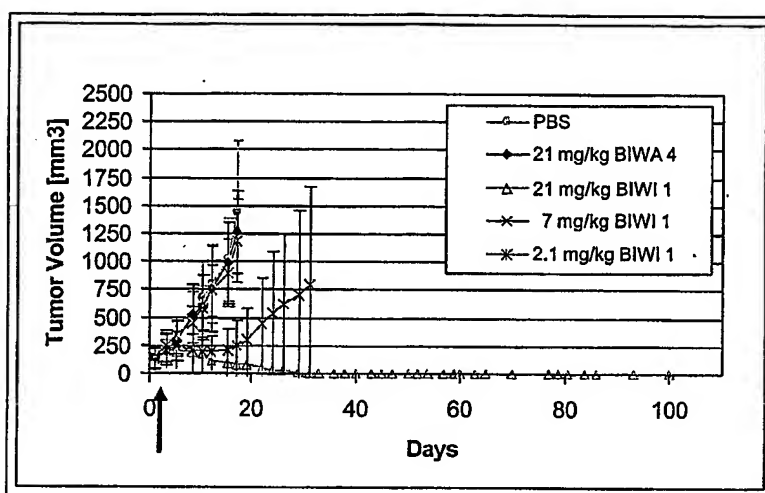


Figure 3

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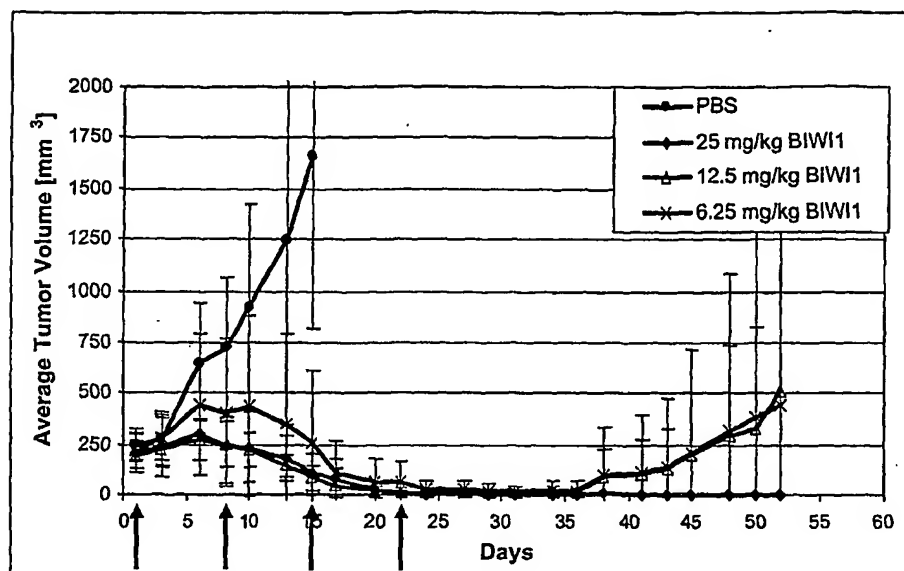


Figure 4



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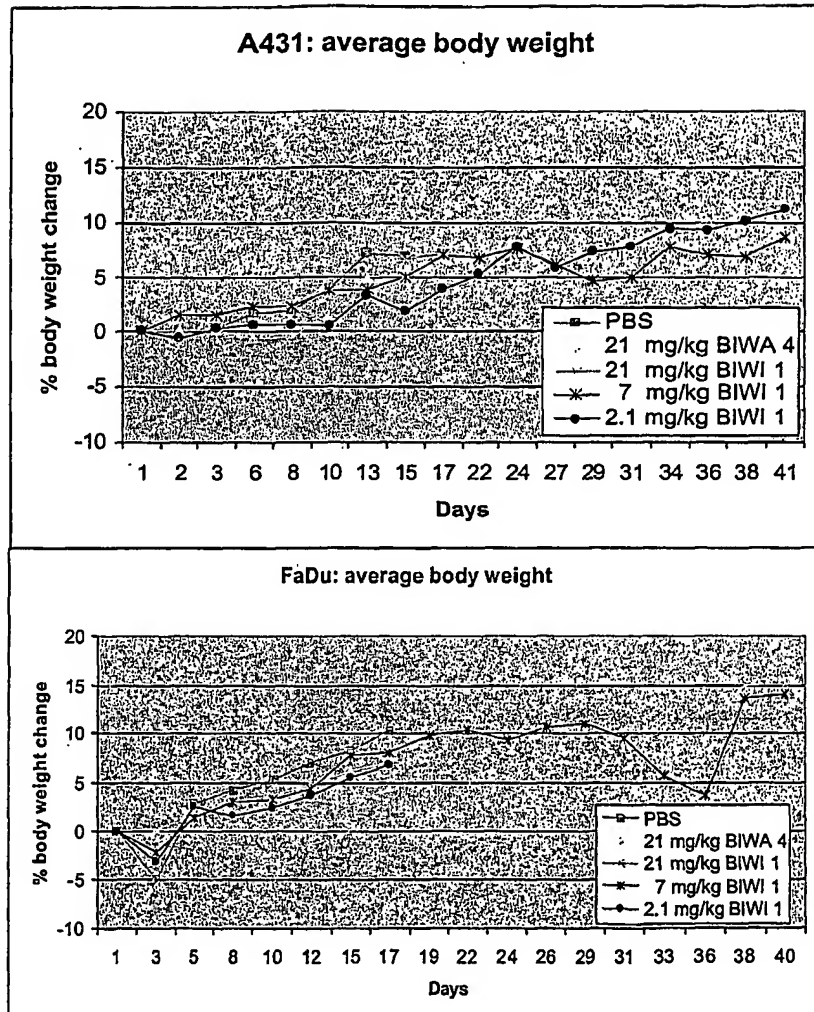


Figure 5

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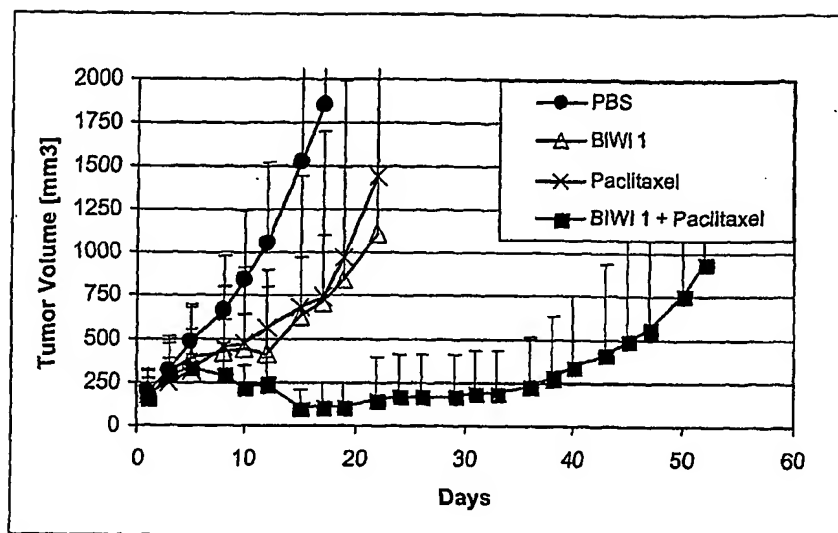


Figure 6

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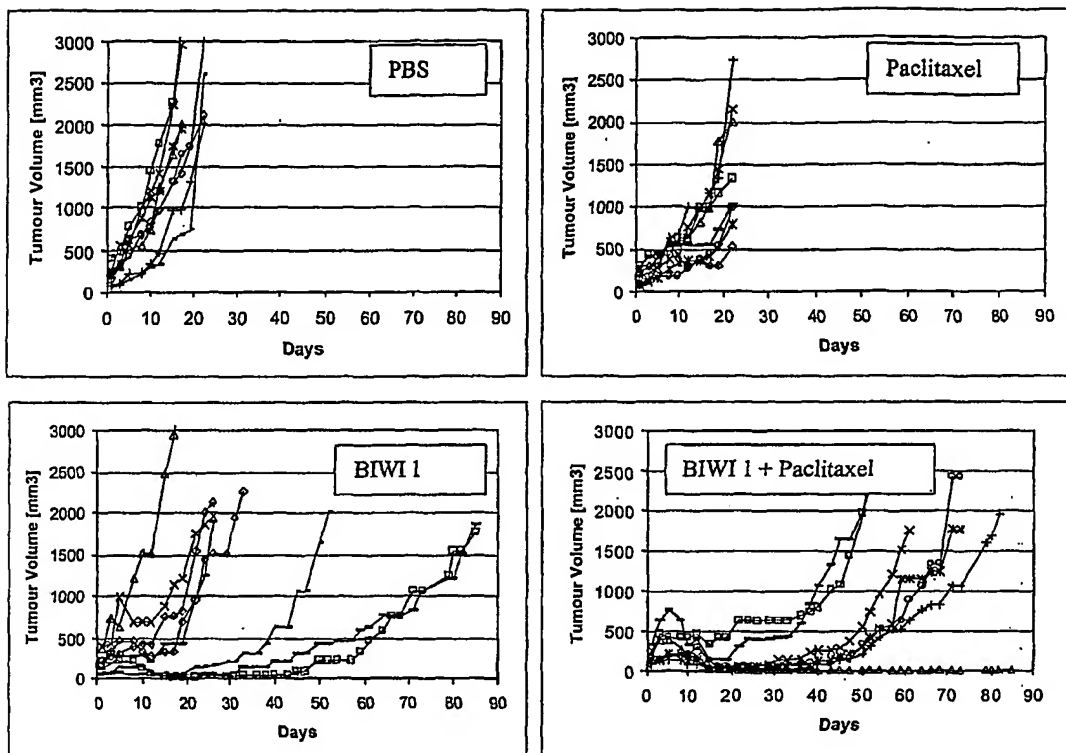


Figure 7

8/8

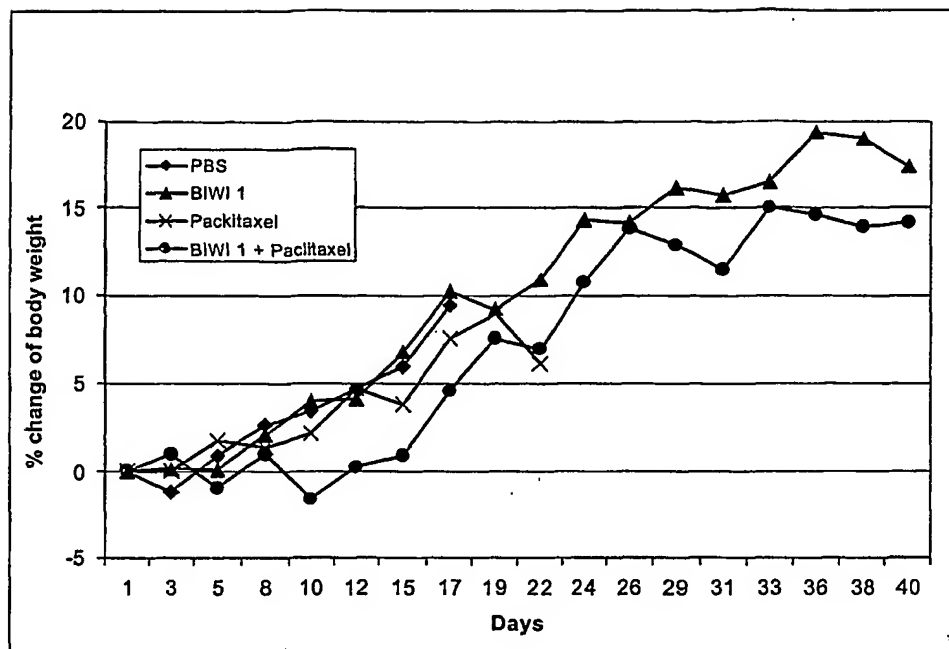


Figure 8

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SEQUENCE LISTING

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<120> Compositions and methods for treating cancer using cytotoxic  
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<140>

<141>

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<170> PatentIn Ver. 2.1

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<212> PRT

<213> Homo sapiens

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<212> PRT

<213> Homo sapiens

<223> Description of Artificial Sequence: SEQ ID NO: 2

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<211> 11

<212> PRT

<213> Homo sapiens

<223> Description of Artificial Sequence: SEQ ID NO: 3

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&lt;210&gt; 4

&lt;211&gt; 213

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Humanised  
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Tyr Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr  
 35 40 45

Leu Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser  
 50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro Glu  
 65 70 75 80

Asp Phe Ala Val Tyr Tyr Cys Leu Gln Trp Ser Ser Asn Pro Leu Thr  
 85 90 95

Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro  
 100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr  
 115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys  
 130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu  
 145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser  
 165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala  
 180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe  
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Asn Arg Gly Glu Cys  
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 35 40 45  
 Ser Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Leu Asp Ser Ile  
 50 55 60  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr  
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 Ala Arg Gln Gly Leu Asp Tyr Trp Gly Arg Gly Thr Leu Val Thr Val  
 100 105 110

Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser  
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 Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys  
 130 135 140  
 Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu  
 145 150 155 160  
 Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu  
 165 170 175  
 Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr  
 180 185 190  
 Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val  
 195 200 205  
 Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro  
 210 215 220  
 Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe  
 225 230 235 240  
 Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val  
 245 250 255  
 Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe  
 260 265 270  
 Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro  
 275 280 285  
 Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr  
 290 295 300  
 Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val  
 305 310 315 320  
 Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala  
 325 330 335  
 Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg  
 340 345 350  
 Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly  
 355 360 365  
 Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro  
 370 375 380  
 Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser  
 385 390 395 400  
 Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln



5

405

410

415

Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His  
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Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys  
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&lt;211&gt; 1392

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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<223> Description of Artificial Sequence: Humanised  
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&lt;211&gt; 213

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

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<223> Description of Artificial Sequence: Humanised  
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6

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 Tyr Trp Leu Gln Gln Lys Pro Gly Gln Ala Pro Arg Ile Leu Ile Tyr  
 35 40 45  
 Leu Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser  
 50 55 60  
 Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro Glu  
 65 70 75 80  
 Asp Phe Ala Val Tyr Tyr Cys Leu Gln Trp Ser Ser Asn Pro Leu Thr  
 85 90 95  
 Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro  
 100 105 110  
 Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr  
 115 120 125  
 Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys  
 130 135 140  
 Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu  
 145 150 155 160  
 Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser  
 165 170 175  
 Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala  
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Murine Antibody BIWA 8 Light Chain SEQ ID NO: 9

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7

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